Atypical Sphingolipids as Biomarkers and Therapeutic Targets in Cardio-metabolic Diseases

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Abstract

Cardiometabolic diseases continue to be a major cause of mortality and morbidity, despite the progress in risk prediction and stratification. Besides the traditional risk factors, sphingolipids are emerging as novel players in the pathogenesis of atherosclerosis and metabolic diseases. Sphingolipid de novo synthesis is typically initiated by the conjugation of L-serine and palmitoyl-CoA, a reaction catalysed by the serine-palmitoyltransferase (SPT). SPT can also metabolise other acyl-CoAs (C_{12} to C_{18}) and other amino acids such as L-alanine and glycine, giving rise to a spectrum of atypical sphingolipids. In a special class of atypical sphingolipids, alanine is conjugated with palmitoyl-CoA, generating 1-deoxysphingolipids (1-deoxySLs) that lack the C_1 hydroxyl group.

In cross-sectional and prospective clinical studies, we explored the potential of these atypical sphingolipids as predictive biomarkers in metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM) and cardiovascular disease. Plasma sphingolipids were extracted, hydrolyzed and the sphingoid base backbones were measured by LC/MS. 1-Deoxy-sphingolipids (1-deoxySLs) were significantly elevated in patients with the metabolic syndrome (MetS) and T2DM compared with controls. In the prospective analysis, baseline plasma 1-deoxySLs were identified as independent predictors for the development of T2DM even after adjustment for HbA_{1c} and the presence of metabolic syndrome. Furthermore, baseline C_{20}SO levels were identified as independent risk predictors for cardiovascular-related events after the adjustment for the traditional risk factors including the degree of coronary artery stenosis.

Pathologically elevated 1-deoxySL levels were found to be associated with hereditary sensory and autonomic neuropathy type 1 HSANI - which is caused by several missense mutations in SPT. The pathological formation of 1-deoxySLs in HSAN1 is significantly suppressed in response to an oral L-serine supplementation in patients and mice models. Given the similarity in the clinical picture of HSAN1 and diabetic sensory neuropathy and the increased plasma levels of 1-deoxySLs in both conditions, we investigated whether a serine supplementation could also be beneficial in the context of the diabetic neuropathy in streptozotocin-treated rats. The effect of serine was tested in preventive and therapeutic schemes. Plasma and tissue sphingolipids were quantified using LC/MS after lipid extraction and hydrolysis. Thermal, mechanical nociception and nerve conduction velocity (NCV) were measured. In both schemes (therapeutic and preventive), plasma 1-deoxySLs were

significantly lowered in the serine-supplemented diabetic rats. Neurologically, the serine supplemented animals showed significantly improved mechanical sensitivity in both the preventive and therapeutic groups, while NCV was significantly better in the preventive but not in the therapeutic group.

We observed consistently a strong positive correlation between plasma 1-deoxySLs and triglycerides (TG). We, therefore, investigated the effect of the triglyceride-lowering drugs, fenofibrate and niacin, on the sphingoid base profile in the plasma of dyslipidemic patients in a multi-center, open label, cross-over study. While fenofibrate and niacin had comparable effects on plasma triglycerides, only fenofibrate lowered significantly the atypical sphingoid bases including 1-deoxySLs. Interestingly, neither fenofibrate nor niacin had any significant effect on the typical sphingolipids (C_{18}-based sphingolipids).

In summary, we showed that 1-deoxySLs are significantly elevated in patients with T2DM and MetS where they could serve as predictive biomarkers for the development of T2DM .We also showed that plasma C_{20}SO is a predictive biomarker for cardiovascular events. We demonstrated that fenofibrate but not niacin was effective in lowering the atypical plasma sphingolipids in dyslipidemic patients. Moreover, an oral L-serine supplementation effectively lowered the plasma 1-deoxySLs in a diabetic STZ rat model and significantly improved the mechanical sensitivity and nerve conduction velocity in these animals. This suggests that oral serine supplementation could be a potential therapeutic strategy in diabetic neuropathy.

Zusammenfassung[1]

Trotz grosser Fortschritte in der Risikovorhersage und der Einteilung der Patienten in entsprechende Risikogruppen, sind Herz und Stoffwechselerkrankungen weiterhin unter den Hauptursachen von Invalidität und erhöhter Sterblichkeit zu finden. Neben den klassischen Risikofaktoren, spielen auch Sphingolipide bei der Pathogenese von Arthrosklerose und anderen Stoffwechselerkrankungen eine immer grössere Rolle.

Die De-novo-Synthese der Sphingolipide wird normalerweise durch die Kondensation von L-Serin und Palmitoyl-CoA, welche durch das Enzym Serin-Palmitoyltransferase (SPT) katalysiert wird, eingeleitet. Die SPT kann jedoch auch andere Acyl-CoA's (C_{12} bis C_{18}) und Aminosäuren, wie etwa L-Alanin und Glycin, als Substrate für dieses Reaktion verstoffwechseln. Diese Substratpromiscuität führt zur Bildung einer ganzen Reihe atypischer Sphingolipide. Die 1-deoxysphingolipide (1-deoxySL), bei denen Alanin mit palmitoyl-CoA konjugiert ist, wodurch es zum Verlust der C_1 Hydroxylgruppe kommt, stellen dabei eine besondere Klasse der atypischen Sphingolipide dar.

In mehrere klinischen Studien untersuchten wir die Verwendungsmöglichkeit dieser atypischen Sphingolipide als prädiktive Biomarker für das Metabolische Syndrom (MetS), den *Typ-2*-Diabetes mellitus (T2DM) und Herz-Kreislauferkrankungen. Die gesammten Sphingolipide wurden aus dem Plasma extrahiert und hydrolysiert. Letztendlich wurden die freien Sphingoid Basen mittels LC/MS identifiziert und quantifiziert. In Patienten mit MetS und T2DM waren die 1-deoxySL im Vergleich zu den Kontrollen signifikant erhöht. Bei der prospektiven Analyse wurden die Ausgangskonzentrationen der 1-deoxySL im Plasma als unabhängige Prädiktoren für die Entstehung von T2DM identifiziert. Diese Assoziation hielt selbst der Adaptierung der Werte an die HbA1c-Werte und das mitunter bereits vorhandene Metabolische Syndrom stand. Des Weiteren konnten wir die gemessenen C_{20}SO-Ausgangswerte selbst nach der Anpassung an an die klassischen Risikofaktoren, sowie den Schweregrad der Herzkranzgefässverengung als unabhängige Risikoprädiktoren für das Auftreten von kardiovaskulären Vorfällen identifizieren.

Es hatte sich herausgestellt, dass pathologisch erhöhte 1-deoxySL-Werte eng mit der hereditären sensorischen und autonomen Neuropathie Typ1 (HSAN1) verbunden sind. HSAN1 wird durch mehrere Fehlsinnmutationen in der Sequenz der SPT verursacht. Die

[1] Translated from the English version of the abstract by ***Heiko Bode***

krankhaft gesteigerte Bildung von 1-deoxySL wird sowohl im HSAN1-Mausmodel, als auch in HSAN1-Patienten durch die orale Verabreichung von L-Serin wirkungsvoll unterdrückt. In Anbetracht der Ähnlichkeit der klinischen Symptome von HSAN1 und der diabetisch sensorischen Neuropathie untersuchten wir weiterhin, ob die Nahrungsergänzung mit L-Serin auch in diesem Kontext hilfreich sein könnte. Diese Untersuchungen wurden mit Ratten durchgeführt, welche durch Behandlung mit Streptozotocin (STZ) zu Diabetikern wurden. Die Wirkung von L-Serin wurde sowohl in einem präventiven, als auch therapeutischen Behandlungsansatz untersucht. Die Sphingolipidzusammensetzung wurde sowohl im Plasma, als auch in den verschiedenen Geweben untersucht und mittels LC/MS nach vorheriger Extraktion und Hydrolyse quantifiziert. Die Schmerzempfindlichkeiten für thermische, und mechanische Reize sowie die Nervenleitgeschwindigkeit wurden ebenfalls gemessen. In beiden Behandlungsansätzen (sowohl präventiv, als auch therapeutisch) führte die Verabreichung von L-Serin zu einer signifikanten Senkung der 1-deoxySL-Konzentrationen im Plasma der diabetischen Ratten. Die mit Serin behandelten Tiere zeigten sowohl in der Präventiv- als auch in der Therapiegruppe eine signifikante Steigerung der mechanischen Empfindlichkeit. Die Nerveneitgeschwindigkeit hingegen konnte nur durch die präventive Gabe von Serin verbessert werden.

Wir beobachteten eine konstante, stark positive Korrelation zwischen den Konzentrationen der 1-deoxySL und den Triglyceridwerten im Plasma**.** Daher untersuchten wir die Auswirkungen der beiden lipidmodifizierenden Medikamente Fenofibrat und Niacin auf das Profil der Sphingolipide im Plasma von dyslipidämischen Patienten. Die Studie wurde als ofene cross-over Studie in mehrere Zentren durchgeführt. Fenofibrat und Niacin zeigten ähnliche Auswirkungen auf Lipoproteine und Apolipoprotein. Lediglich Fenofibrat hingegen konnte die Konzentrationen aller atypischer Sphingolipide inclusive der 1-deoxySL signifikant senken, dabei wurden die gemessenen Konzentration der typischen Sphingolipide (aus C_{18}-Sphingoidbasen aufgebaut) weder durch Fenofibrat, noch Niacin signifikant verändert.

Zusammenfassend konnten wir zeigen, dass 1-deoxySL sowohl in T2DM- als auch in MetS-patienten in signifikant erhöhten Konzentrationen gemessen werden können. Im Falle des metabolischen Syndroms können sie daher als prädiktive Biomarker für die Entstehung von T2DM herangezogen werden. Des Weiteren zeigten wir, dass die Konzentration von C_{20}SO im Plasma ein prädiktiver Biomarker für das Auftreten kardiovaskulärer Ereignisse ist. Wir demonstrierten, dass Fenofibrat, jedoch nicht Niacin, die Konzentrationen der atypischen

Sphingolipide im Plasma von dyslipidämischen Patienten wirkungsvoll senken kann. Darüber hinaus senkte die orale Verabreichung von L-Serin die 1-deoxySL-Konzentrationen im Plasma von diabetischen STZ-Ratten und verbesserte zeitgleich sowohl deren Empfindlichkeit für mechanische Reize als auch die Nervenleitgeschwindigkeit. Dies lässt darauf schließen, dass die orale Verabreichung von L-Serin eine potentielle Therapiemöglichkeit für die Behandlung der diabetischen Neuropathie sein könnte.

Introduction:

Sphingolipids: More than a century of biochemical pathways and not yet totally deciphered

"Enigmatic by nature" is the best fitting and most frequently used description of sphingolipids. The term "sphingosine" was first used by J.L.W. Thudichum (1829 – 1901) in his book "A treatise on the chemical constitution of the brain"[1], after being inspired by the Greek myth about the Sphinx, and in his words "in commemoration of the many enigmas which it presented to the inquirer".

De novo sphingolipid biosynthesis (Fig. 1) usually starts in the ER with the condensation of serine and palmitoyl-CoA generating 3-ketosphingaine. This, first and rate limiting, reaction is catalyzed by the enzyme serine palmitoyltransferase (SPT) [2-5], a pyridoxal 5′-phosphate (PLP)-dependent enzyme which belongs to the subfamily α-oxoamine synthase. SPT is likely a multimeric protein complex with a molecular weight of 460-480kDa [6]. Three subunits, SPTLC1, SPTLC2 [2, 3, 5] and SPTLC3 [7], have been identified as part of this complex. Besides serine and palmitoyl-CoA, SPT can also use other acyl CoAs (C_{12}-C_{18}) [8] and other amino acids such as alanine and glycine as alternative substrates [9, 10]. The canonical (typical) reaction with serine and palmitoyl CoA forms sphingoid bases with a C18 carbon chain length (C_{18}-based sphingolipids) whereas the use of other acyl CoAs results in the formation of sphingoid bases with a different carbon chain length. The use of alanine or glycine instead of serine generates, in contrast, an atypical category of 1-deoxysphinoglipids (1-deoxySL) which lack the C_1 hydroxyl group.

The direct SPT product, 3-keto-sphinganine, is rapidly reduced to sphinganine (SA) by the NADPH-dependent enzyme, 3-keto-sphinganine reductase (KSR) [11]. Typically, SA is *N*-acylated by the enzyme ceramide synthase (CerS) to form dihydroceramides [12]. CerS comprises a family of related enzymes (CerS1-6), each of which has a different tissue distribution and a distinct specificity for certain fatty acid substrates [12]. This generates a combinatorial diversity since distinct fatty acids, differing in the carbon chain length (C_{12}-C_{26}), hydroxylation and the number of double bonds, can be attached to the sphinganine backbone. In a minor pathway, SA can also be phosphorylated at C_1 by the enzyme sphingosine kinase (SphK) [13] to form sphinganine 1-phosphate (SA1P).

Ceramides [14, 15] are formed subsequently by introducing a *trans*-double bond into the SA backbone of the dihydroceramide at the C_4-C_5 position. This desaturation step is catalyzed by

the enzyme Δ4-dihydroceramide desaturase (DES) which uses NADH or NADPH as an electron donor and oxygen as an electron acceptor in a series of NADH-FADH-cytochrome b_5 electron transfer reactions [14]. Two isoforms of the mammalian dihydroceramide desaturases are characterized which appear to have different functions. While DES1 is primarily responsible for introducing the double bond, DES2 can hydroxylate the C_4 of the sphinganine backbone which then forms phytoceramide [16].

Ceramides are the central branching points in sphingolipid de-novo synthesis and represent a crossing point for the degradation and salvage pathways. Ceramides are one of the major bioactive sphingolipids with diverse roles in intracellular and extracellular signaling [17]. Notably, all the enzymes involved in the de-novo synthesis of ceramides are located at the cytosolic surface of the ER [18] [14]. For further metabolism, ceramides have to be transported from the ER to the Golgi either through vesicular or non-vesicular transport. Ceramide transfer protein (CERT) is responsible for the non-vesicular transport of ceramides from the ER to the Golgi compartment [19]. In the Golgi, ceramides can be converted to sphingomyelins by sphingomyelin synthase (SMS) [20, 21]. Sphingomyelin synthase catalyzes the exchange of a phosphocholine group from phosphatidyl choline to the C_1 hydroxyl group of ceramides thereby forming sphingomyelin and diacylglyerol in the same reaction. There are three sphingomyelin synthases - SMS1, SMS2 and SMSr. While SMS1 is present on the lumenal surface of the Golgi, SMS2 is located within the plasma membrane [22]. Additionally, the sphingomyelin synthase-related enzyme (SMSr) is present in the ER where it exchanges a phosphoethanolamine group from phosphatidyl ethanolamine to ceramides generating ceramide phosphoethanolamine [22, 23]. SMSr has been suggested to play a role in ceramide homeostasis in the ER [23].

Ceramides can be glucosylated in the Golgi by GlcCer synthase (UDP-Glc:ceramide glucosyltransferese) to form glucoceramide (GlcCer). GlcCer synthase attaches one glucose moiety to the C_1 hydroxyl group of ceramides forming a β glycosidic bond to the C_4 hydroxyl group of glucose (4Glcβ1-Cer) [24]. In contrast to SMS1, GlcCer synthase is located at the cytosolic side of the Golgi [25]. For GlcCer to be further metabolized, the recently discovered transport protein FAPP2 (four-phosphate adaptor protein) [26] is essential to transfer the GlcCer from the cytosolic surface of the Golgi to the luminal side.

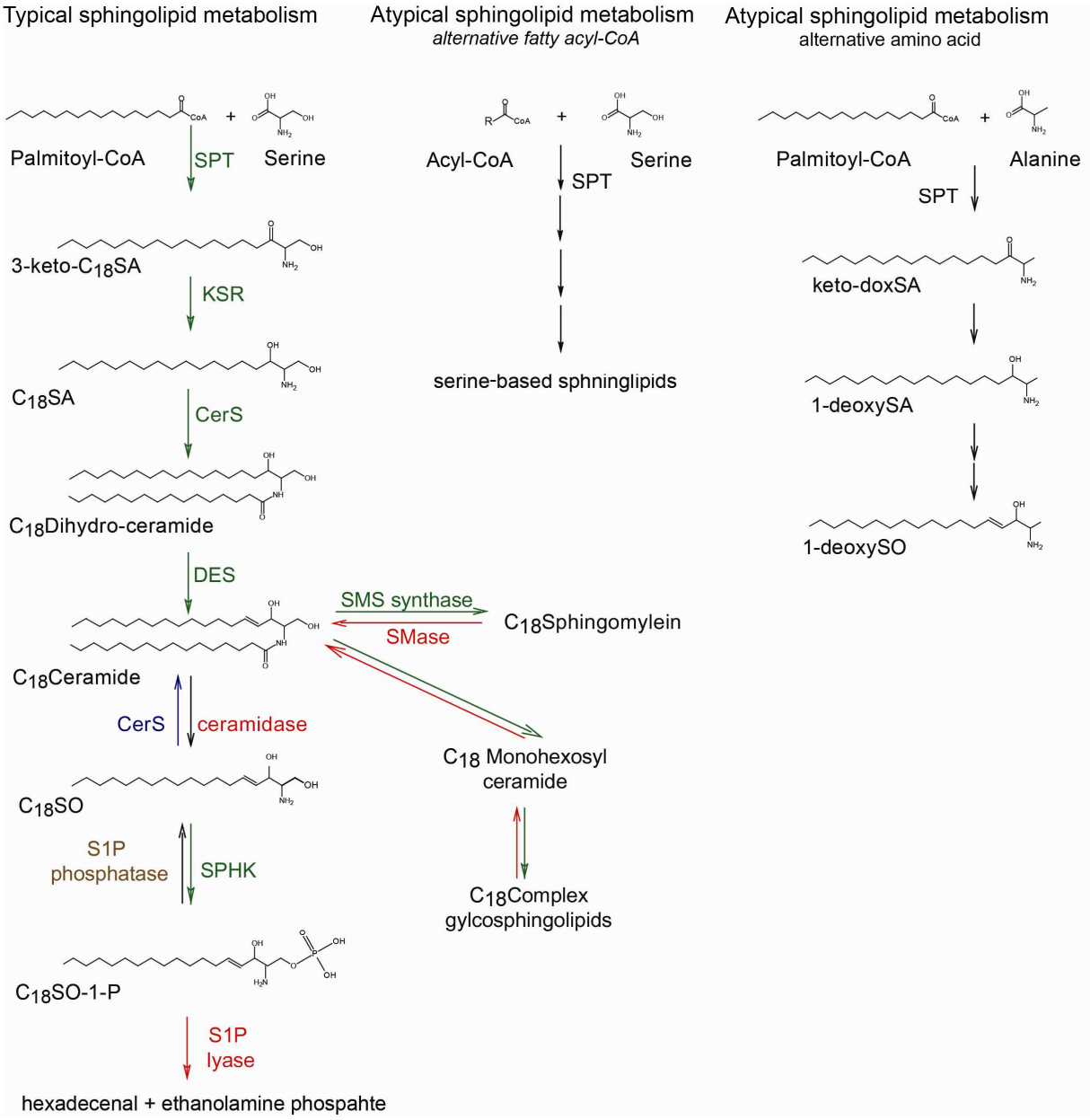

Figure 1. Sphingolipid metabolism pathways. Overview of the major enzymes and reactions in typical sphingolipid metabolism using the canonical substrates (serine and palmitoyl-CoA and in atypical sphingolipid metabolism using alternative acyl-CoAs and amino acids (alanine). green color represent the biosynthetic pathway while red color represents the degradation pathway SPT, serine palmitoyltransferase, CerS, ceramide synthase, SPHK, sphingosine kinase, S1P, sphingosine 1-phosphate,KSR, ketosphinganine reductase, DES, dihydroceramide desaturase,1-deoxySA,1-deoxysphinganine,1-deoxySO,1deoxysphingosine, R, an alkyl chain with the length (C_{10} to C_{20})

Complex gylcosphingolipids are then formed on the luminal side of the Golgi by the sequential addition of different sugar moieties (glucose or galactose), sialic acid and hexosamine (glucosamine or galactosamine).

Besides glucose, ceramides are also conjugated to galactose by the enzyme GalCer synthase (UDP-Gal:ceramide galactosyltransferese) to form galactosyl-ceramide (GalCer) [27-29].

GalCer are mainly present in the nervous systems of mammals. Interestingly, GalCer synthase is an integral membrane protein of the ER [30], where the active site is located on the lumenal side of ER. This indicates that ceramides produced in the ER by the de novo pathway have to flip to the lumenal side to form GalCer. GalCer are transported to the Golgi and then to the plasma membrane. Along the way they can be sulfated forming sulfatides [31].

Gylcosphingolipids and sphingomyelin are transported from the Golgi to the plasma membrane mostly by vesicular transport along the exocytic or endocytic recycling pathways [32]. It is noteworthy, that the pathway for the de novo synthesis of sphingolipid is well aligned with the secretory pathway (ER-Golgi-plasma membrane).

Ceramides can also be phosphorylated in a reaction catalyzed by the ceramide kinase to form ceramide1-phosphate (Cer1-P) [33, 34]. Cer1-P has been suggested to promote proliferation and to play a role in inflammation [35].

Sphingomyelinase (SMase) is responsible for the degradation of sphingomyelin into ceramide. Three SMases have been identified; acid, alkaline and neutral sphingomyelinase [36-38], depending on the optimum pH for their activity. Acid SMase exists as both a lysosomal and a secreted enzyme while alkaline SMase is secreted in the gut. Neutral sphingomyelinase represents a family of enzymes which reside in the ER, Golgi, plasma membrane or nucleus where they are suggested to play different biological functions in the local sphingolipid homeostasis within these compartments [39].

The degradation of gylcosphingolipids happens in the lysosome in a stepwise fashion by releasing one carbohydrate moiety at a time until gulcosylceramidase hydrolyses GlcCer into ceramides in the last step [40]. Non-lysosomal degradation of gylcosphingolipids (e.g. on the plasma membrane) is also reported [41]. Ceramides formed from gylcosphingolipids or sphingomyelin are finally hydrolyzed by ceramidase to form sphingosine and a free fatty acid, both of which can leave the lysosome in contrast to ceramides [42]. Five different ceramidases have been identified: acid ceramidase, neutral ceramidase and alkaline ceramidase 1, 2 and 3. They reside in the lysosome, plasma membrane and ER probably playing distinct roles in maintaining an appropriate sphingosine to ceramide balance within these compartments [42]. The sphingosine (SO) released from ceramides differs from sphinganine (SA) by the presence of a C_4-C_5 *trans* double bond. SO is mainly released from the lysosome but can also be generated in the ER, Golgi or plasma membrane depending on the action and tissue distribution of the various ceramidases [42]. SO can be reacylated by

CerS to reform ceramides [43]. Alternatively, SO can be phosphorylated by sphingosine kinases (SphK) to form sphingosine 1-phosphate (S1P). There are two known isoforms of sphingosine kinases, SphK1 [44] and SphK2 [45] which are mostly located in the cytosol and differ in tissue distribution and biochemical properties [46]. S1P is a highly bioactive lipid which acts as an intracellular and extracellular ligand for five G-coupled receptors (S1PR1-5). S1P can be de-phosphorylated by S1P phosphatase to form SO again or irreversibly degraded by S1P lyase [47] to hexadecenal and ethanolamine phosphate. This final degradation step occurs at the cytosolic surface of the ER [48]. Thus, the entry and the exit points of the sphingolipid metabolism are both located at the ER.

Three major observations can be made by studying sphingolipid metabolism. First, the vast combinatorial diversity in sphingolipid molecules which arise by the combination of different sphingoid bases with different N-acyl chains and various headgroups. In theory, these permutations can form thousands of individual sphingolipid subspecies [49], although it is not clear whether all these species are really present in biological systems. However, already in human plasma > 200 distinct sphingolipid species have been identified [50]. Second, the subcellular localization and compartmentalization of the metabolizing enzymes point at distinct local pools for each compartment and the need for an intricate balance between synthesis and degradation within each compartment and within the whole cell. Third, most of the above mentioned pathways, if not all, were studied by focusing on the canonical pathway, i.e. on C_{18}-based sphingolipids. Little is known about the metabolism, regulation and transport of sphingolipids with different backbones. It is therefore largely unknown whether these metabolites follow the same metabolic pathways or whether they have their own specific enzyme machinery.

Sphingolipids and disease:

We will focus here on the mutations affecting different enzymes of sphingolipid metabolism leading to monogenic disease, and the role of different sphingolipids metabolites in cardiometabolic diseases

Monogenic inborn errors of sphingolipid metabolism

Most disease causing mutations in the sphingolipid metabolism affect enzymes involved in the degradation of complex sphingolipids (gylcosphingolipids and sphingomyelins), ceramides or essential co-factors such as sphingolipid-activator proteins (SAPs) which are required for the hydrolysis of certain gylcosphingolipids (Fig. 2) [51]. Since the breakdown of

sphingolipids occurs mainly in the lysosomes, these defects in sphingolipid catabolism are typically associated with lysosomal storage disorders - collectively termed as sphingolipidoses. Sphingolipidoses are inherited as autosomal recessive disorders with the exception of Fabry`s disease which is X-linked [52]. The clinical presentation is heterogeneous and depends mainly on the metabolic flux through the defective enzymes and their tissue distribution. GM1 gangliosidosis, Tay-Sachs and Sandhoff disease are characterized by the accumulation of gangliosides [53, 54] leading primarily to neurological manifestations as well as to an enlargement of spleen and liver. Fabry`s disease is caused by defective action of the α-galactosidase enzyme leading to accumulation of neutral glycosphingolipids in the vascular endothelium of the heart, kidney and nervous system [55]. This in turn leads to cardiac, renal and neurological impairment in these patients [55].

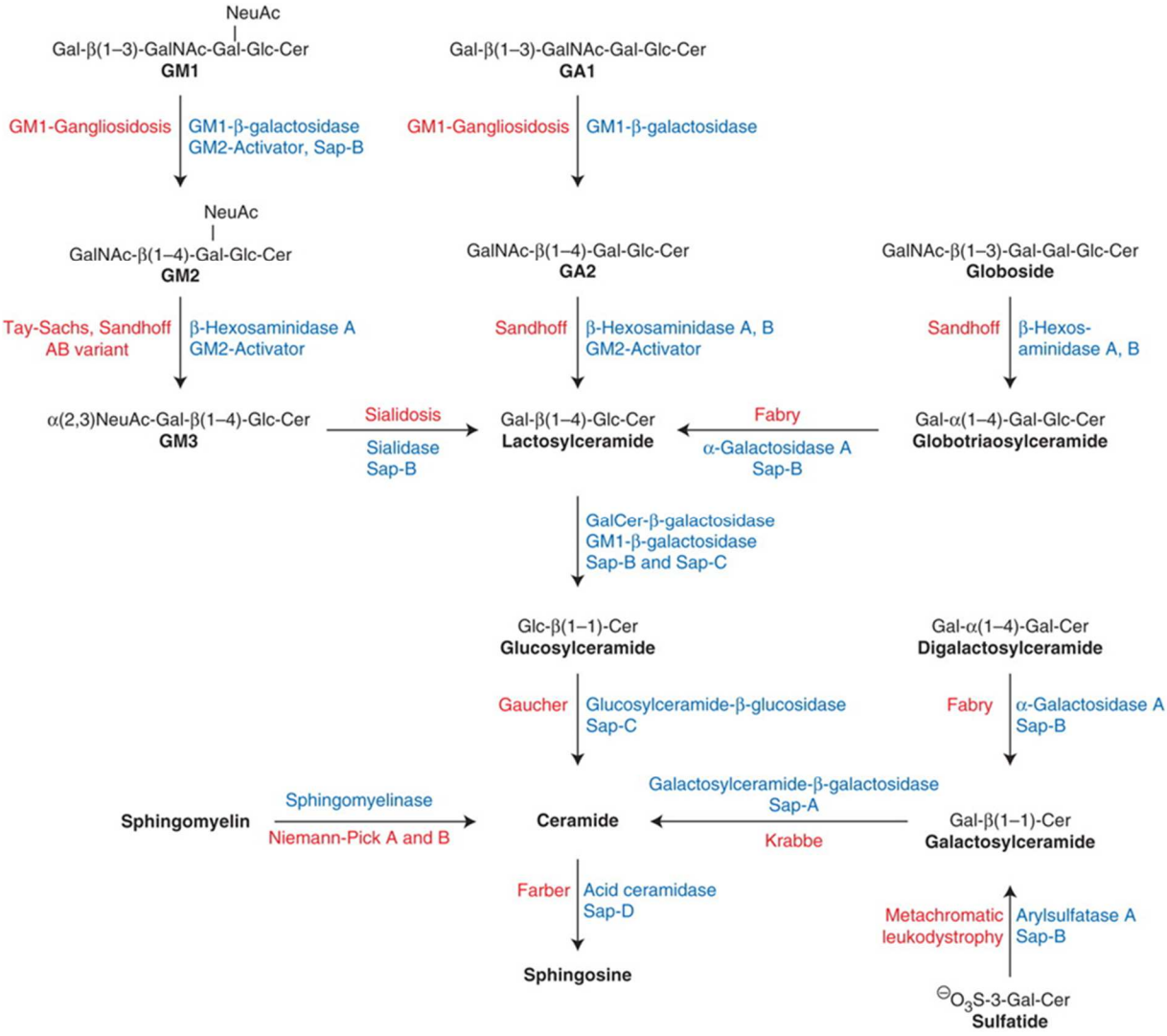

Figure 2. Enzymatic defects in sphingolipid lysosomal storage diseases (sphingolipidoses). Each disease is mapped to the enzyme defect responsible for the accumulation of its substrate. Reproduced from [51]

In Gaucher disease, which is the most common sphingolipidoses, the accumulation of glucosylceramide happens to a large degree in the macrophages of the reticulo-endothelial system leading to hepato-spleenomegaly and blood disorders. In some variants of Gaucher disease, the accumulation of glucosyl-sphingosine and not of glucosylceramide is the cause of the disease which then leads to primarily neurological manifestations [56, 57].

In metachromatic leukodystrophy, the accumulation of sulfatides affects mostly the myelin sheath [58] in the white matter of the brain and peripheral nerves leading to a progressive demyelination and a dysfunction of the central and peripheral neurons [58]. Farber`s disease is characterized by acid ceramidase deficiency and an accumulation of ceramides in the lysosomes [59, 60] which affects many organs and results in a typical skin phenotype. Niemann–Pick disease (NPD) type A and B is caused by defects in acid sphingomyelinase which leads to an accumulation of sphingomyelin and sphingosyl-phosphocholine in the lysosomes and other organelles [61]. Patients with NPD usually present with hepato-splenomegaly and impairments in the nervous system. Interestingly, NPD type C is caused by mutations in the two proteins (NPC 1 and 2) that are involved in the lysosomal transport of cholesterol [62] but leads also to an accumulation of sphingomyelin which indicates an association between sphingolipid and sterol metabolism [63].

Interestingly, almost all catabolic enzymes in sphingolipid degradation are associated with a genetic disease, while only two diseases have been associated with enzymes in the sphingolipid biosynthetic pathway. This is the infantile epileptic syndrome and hereditary sensory and autonomic neuropathy type I (HSAN1). Infantile epileptic syndrome has been reported recently to be caused by GM3 synthase deficiency which leads to an increase in lactosyl-ceramide, the substrate for GM3 synthase [64]. Mutations in the SPT, the first and rate limiting enzyme for sphingolipid biosynthesis, have been reported to cause HSAN1 [65-68]. HSAN1 is an autosomal dominant genetic disease which affects mainly the peripheral sensory nerves and to a variable extent the motor and autonomic nerves. HSAN1 mutations in SPT were shown to increase the activity of SPT to use alanine and glycine as alternative substrates [9, 69], which leads to an increased generation of 1-deoxySLs. 1-deoxySLs are toxic to neurons in vitro and were shown to affect neurite length and branching in chicken dorsal root ganglia (DRGs) [70].

Sphingolipids and cardiovascular disease:

Cardiovascular disease comprises a broad spectrum of heterogeneous disorders affecting the heart and blood vessels. This includes atherosclerotic cardiovascular disease, congenital heart disease, rheumatic heart disease and others. Atherosclerotic cardiovascular disease affects the large and medium-sized arteries leading to coronary artery disease (CAD), cerebrovascular disease or peripheral artery disease. Atherosclerosis is a chronic process where lipids accumulate over decades within plaques in the arterial wall leading to a narrowing of the lumen. Severe narrowing or plaque rupture compromises the blood flow to the affected organs and presents clinically as acute pain or dysfunction of the affected organ. Limiting the blood flow in the coronary arteries leads to chest pain (angina) while a complete occlusion, usually by a thrombus of one of the arteries, leads to an ischemic damage of the heart muscle (myocardium) and myocardial infarction (MI). A similar situation in the brain leads to transient ischemic attacks (TIAs) or stroke. In severe conditions, where the heart or the brain function is largely compromised, death can occur. Atherosclerotic cardiovascular disease represents one of the major health problems worldwide causing substantial morbidity and mortality and affects significantly the quality of life [71-74].

Several lipid classes accumulate in the arterial wall and it has been shown in several cohorts over decades that an altered plasma lipoprotein profile is one of the major risk factors to develop MI or stroke [75]. Of these lipid classes, sphingolipids are increasingly recognized as mediators in the pathogenesis of atherosclerotic cardiovascular disease [76-78]. In particular, the role of sphingomyelin, ceramides and sphingosine 1-phosphate in atherosclerosis has been extensively studied. Conversion of sphingomyelins to ceramides by soluble acid SMase in the LDL particles was shown to increase LDL particle aggregation in the arterial wall and to promote atherosclerosis [79, 80]. S1P was shown to play a pro- and anti-atherogenic role, thus the role of S1P in atherosclerosis remains a matter of debate [81, 82]. It cannot be excluded that the pro- and anti-atherogenic effects of S1P depend on the functional context (e.g. source and carrier of S1P in plasma, expression of S1P receptor isoforms and other lipids that might interfere with its function). Plasma sphingomyelin (SM) was found to be significantly elevated in patients with CAD [83, 84] and to be an independent predictor of MI and cardiovascular death in acute coronary syndrome patients [85]. Moreover, oral administration of myriocin, a potent inhibitor of SPT, decreased plasma SM, total cholesterol, triglycerides and increased HDL cholesterol and resulted in reduced atherosclerotic lesions in ApoE knockout mice [86]. Mechanistically, it has been suggested that plasma SM plays a role in

regulating the activity of lecithin-cholesterol acyltransferase (LCAT) [87] and it was shown that SPTLC2-deficient mice and SM synthase-deficient mice have elevated LCAT activities which was suggested to result from the decrease in plasma sphingomyelin. Interestingly, patients with mutations in the enzyme acid SMase (Niemann Pick disease type B) were found to have significantly lower plasma HDL-C levels and elevated triglycerides. It has been suggested that HDL maturation is impaired in these patients due to the inhibitory effect of SM on LCAT activity [88]. This suggests that plasma sphingomyelin plays a role in lipoprotein metabolism, which might explain its relation to coronary artery disease.

Sphingolipids, T2DM and the metabolic syndrome:

Diabetes mellitus (DM, *Latin for* sweet urine) represents one of the major health problems worldwide contributing substantially to mortality and morbidity and affecting the quality of life of millions of patients [71-74]. DM is diagnosed by elevated plasma glucose levels (hyperglycemia) either after fasting or 2h hours after an oral glucose challenge [89]. There are two major distinct clinical and pathological types of diabetes mellitus, DM type 1 (T1DM) and DM type 2 (T2DM). In T1DM, hyperglycemia occurs due to the loss of pancreatic β islets as a result of an autoimmune reaction leading to a significant drop in plasma insulin levels within a few days to weeks which finally results in absolute insulin deficiency. On the contrary, T2DM is a multifactorial disease with several genetic and environmental factors involved. Insulin resistance is one of the hallmarks of T2DM pathogenesis which leads to hyper-insulinemia as a compensatory mechanism. After several years of hyperinsulinemia, β cell failure occurs in a group of patients leading to impaired glucose tolerance and overt T2DM [90]. T2DM leads to various macrovascular (e.g. MI and stroke) and microvascular complications such as diabetic retinopathy and nephropathy as well as peripheral neuropathy. Insulin resistance tends to cluster in the same patients together with other risk factors for cardiovascular disease such as dyslipidemia and hypertension. This accumulation of these risk factors is usually referred to as the “metabolic syndrome (MetS)” [91, 92].

A unified model for the pathogenesis of insulin resistance is still elusive, despite the considerable progress in understanding the different molecular mechanisms [93, 94]. A growing body of evidence suggests that sphingolipids contribute to the pathogenesis of insulin resistance and diabetes [95-98]. In particular, ceramides have been shown to be involved in the development of insulin resistance. Plasma ceramides were found to be elevated in T2DM patients [99] and to correlate positively with insulin resistance. Furthermore, ceramides have been shown to counteract insulin action on glucose uptake and glycogen synthesis by

inhibiting protein kinase B/Akt through different mechanisms [100]. Recently, it was demonstrated that many of the beneficial effects of adiponectin on insulin sensitivity could be attributed to its lowering effects of intracellular ceramides [101]. Other sphingolipids such as glycosphingolipids may also play a role in T2DM [102]. For instance, it was shown that plasma gylcosphingolipids are significantly altered in diabetic monkeys. It was also reported that mice lacking the ganglioside GM3 had improved insulin sensitivity [103, 104]. Moreover, inhibiting SPT with myriocin was shown to improve insulin resistance and to preclude the development of T2DM in animal models [105].

Novel biomarkers: unmet needs in Cardio-metabolic disease

Cardiometabolic diseases such as T2DM and atherosclerotic CVD are classical examples for complex multifactorial diseases where several factors inter-play to produce a heterogeneous phenotype. Despite of a variety of risk prediction algorithms and biomarkers, a considerable number of patients cannot be assigned to their appropriate risk categories. At least 15-20 % of the CAD patients cannot be predicted by any of the traditional risk factors [106]. Similarly, impaired fasting glucose and impaired glucose tolerance fail to predict almost 50% of the patients who will develop T2DM [107, 108]. These patients cannot be subjected to primary prevention as they do not fit into the current risk classification schemes. This underscores the need for novel biomarkers that allow the identification of those patients who are at risk despite the absence of classical risk factors and biomarkers [109, 110]. Moreover, there is a clear need for biomarkers to stratify groups of diabetic patients with an increased risk to develop macrovascular or microvascular complications [111, 112].

In general, a biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (NIH biomarkers definitions working group) [110, 113]. According to this definition, biomarkers can be obtained by measuring biological samples (e.g. plasma or other body fluids), by performing a specific test on the patients (e.g. hypertension) or through imaging techniques. Biomarkers can be used to identify the risk to develop the disease (risk factor or risk marker), to detect the subclinical disease (screening biomarkers), to predict the future development of the disease (prognostic biomarkers) or can be used for disease staging and diagnosis [113].

For a biomarker to be successfully applied in the routine clinical practice, five different phases of biomarker development have been suggested [114, 115]. In phase I, potential

biomarkers are discovered either by hypothesis-driven approaches or hypothesis free approaches (-omics technologies). Phase II entails the clinical assay development and validation where patients with the disease are compared to controls in a case-control design. A retrospective analysis of stored samples is performed in phase III of biomarker development while in phase IV, the analysis is done in prospective studies where patients are followed over a specific period of time after the biomarker has been assessed at baseline. In the final phase V, an interventional trial is performed to evaluate the benefits of measuring the biomarker on the patients` outcome [110, 114].

Plasma sphingolipids:

Plasma sphingolipids constitute about 4% of total plasma lipids but comprise > 200 distinct species [50, 116]. This diversity is generated by attaching different sphingoid base backbones to different fatty acids in the ceramide moiety combined with different head groups in complex sphingolipids. Sphingomyelins are the major sphingolipid species in plasma representing ~95% of total plasma sphingolipids. Ceramides and monohexosyl ceramides are minor species and represent 3.5 % and < 1% of total plasma sphingolipids, respectively, (Fig. 3) [50, 116]. Considering the sphingoid base profile, C_{18}SO is the most abundant representing ~60% of the total sphingoid base backbones. C_{18}SAdiene, C_{18}SA and C_{16}SO represent 18%, 9% and 10% respectively. Other sphingoid bases are present to a minor extent (< 5%) (Fig. 3) [50, 116]. 1-deoxySLs are present in the plasma of healthy individual but reflect less than 1% of the total plasma sphingolipids) [9, 117]. Sphingolipids in plasma are primarily transported within the different lipoprotein fractions [118]. VLDL and LDL contain higher amount of the plasma sphingomyelin and ceramides compared to HDL [118]. S1P was shown to bind specifically to ApoM in HDL particles and it was suggested that ApoM is the specific carrier for S1P in plasma [119]. Interestingly, plasma 1-deoxySLs are found only in the VLDL and LDL but not in the HDL fraction of plasma of healthy subjects [117].

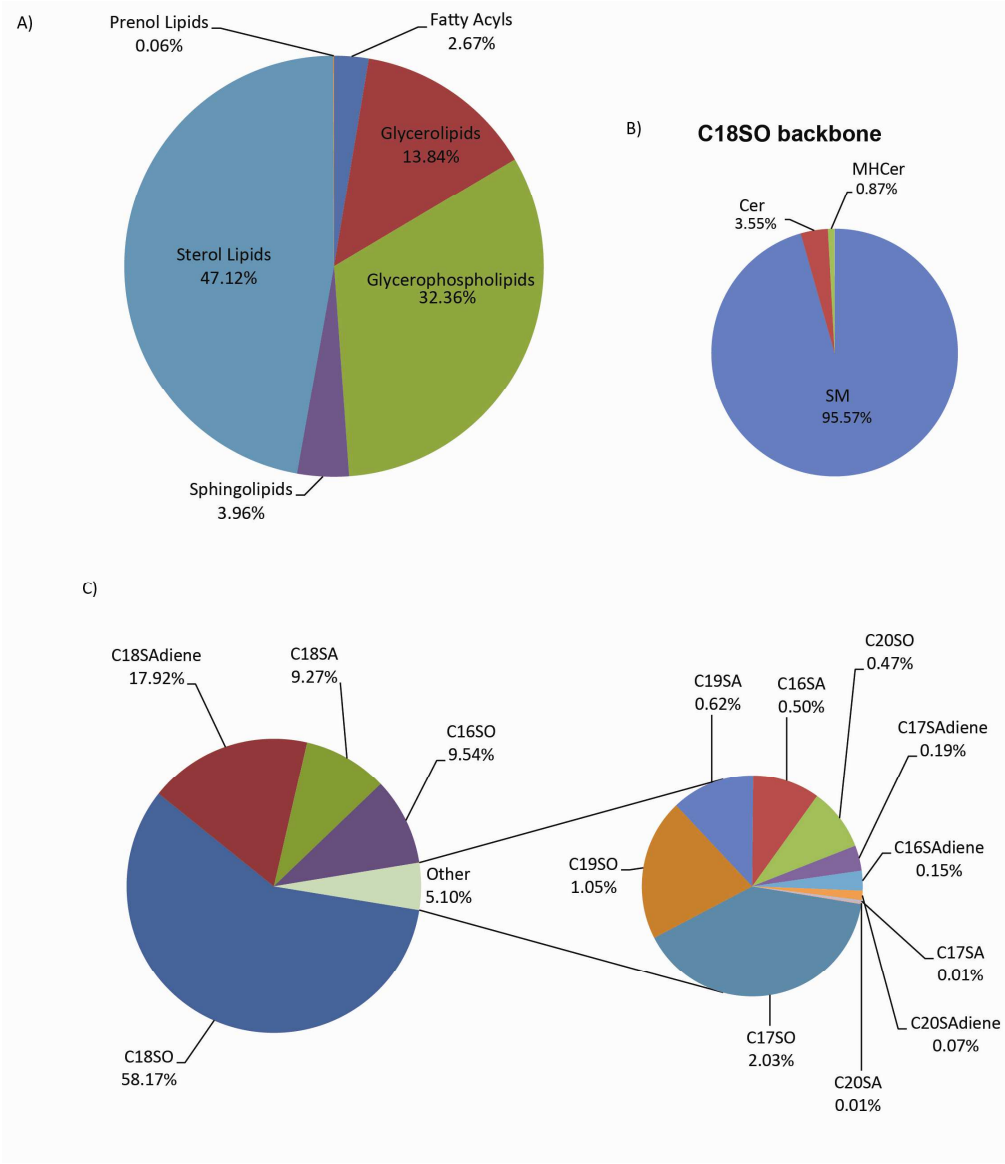

Figure 3. Distribution of plasma lipids (A) and sphingolipids (B-C). Plasma sphingolipids distribution is shown by the headgroup species for C18 sphingoid base backbone (B). The sphingoid base backbone distribution of total plasma sphingoid backbones (C). The figure is reproduced from the data published in [50]

1-deoxysphingolipids: from marine chemotherapeutics to targets in a rare hereditary neuropathy

As mentioned above, serine palmitoyltransferase (SPT) shows promiscuity for its substrates under certain conditions. It can use acyl-CoAs other than palmitoy-CoA and amino acids other than serine producing atypical sphingolipids. In the case of alanine and palmitoyl CoA, SPT produces a special class of 1-deoxysphingolipids (1 deoxySLs) that lack the C_1 hydroxyl group. Since the C_1 hydroxyl group is required for the formation of complex sphingolipids and degradation, 1-deoxySLs cannot be metabolized to complex sphingolipids nor degraded

through the canonical pathway. Thus, they represent dead-end metabolites. Initially, the compound 1-deoxySA was first discovered in the marine clam *Spisula polynyma* through a screen for marine-based cancer chemotherapeutics [120]. It was given the name ES-285 (according to its MW of 285.3) or spisulosine after the clam in which it was discovered [120]. ES-285 was shown to be cytotoxic in mammalian cell culture inducing characteristic loss of actin stress fibers [120]. This cytotoxic effect has been suggested to be mediated through the activation of an atypical PKC isoform (PKC ζ) [121, 122]

We [9] and others [10] have identified 1-deoxySA as a direct product of the SPT reaction which is formed by the condensation of alanine and palmitoyl-CoA. It was shown that the ceramide synthase inhibitor Fumonisin B1 (FB1) causes an increased formation of 1-deoxySA in mammalian cell lines [10]. It was also demonstrated that the prolonged culture of certain mammalian cell lines leads to accumulation of 1-deoxySLs especially, 1-deoxydihydroceramides. However, it is not understood why the SPT increases its utilization of alanine instead of serine, in these conditions but this remains an area of active investigation.

Several missense mutations in the SPT lead to increased activity with alanine and glycine resulting in increased 1-deoxySLs formation in patients with hereditary sensory and autonomic neuropathy (HSAN1). HSAN1 is an autosomal dominant disease which affects mainly the peripheral sensory nerves and to a variable extent the motor and autonomic nerves. Clinical symptoms start typically in the 2nd to 3rd decade of life with the loss of sensations for pain, temperature and vibration in the feet and hands. The disease is slowly progressing in a stocking-and-glove distribution. Sensory loss leads to ulcers and mutilations which might require amputations. Positive sensory symptoms such as parasthesias or lancinating pain, motor or autonomic symptoms are also frequently present [123, 124]. It was shown that 1-deoxySLs are neurotoxic and affects neurite formation, length and branching in cultured chicken dorsal root ganglia (DRGs) [70]. There is a surprising similarity in the clinical symptoms between HSAN1 and the diabetic sensory polyneuropathy (DSN) - the most common form of the diabetic neuropathy [125]. DSN usually starts in the lower extremities with either negative (e.g. numbness) or positive symptoms (e.g. neuropathic pain) or a combination of both. Symptoms are also symmetrical on both sides with a “stocking and gloves” distribution. Sensory loss is frequently associated with bad wound healing at pressure

points and ulcers. This in turn frequently requires amputations leading to a significant reduction in the quality of life [126].

We showed previously that an oral serine supplementation results in a significant lowering of plasma 1-deoxySL. This was demonstrated in transgenic HSAN1 animal models and in a pilot study with HSAN1 patients [127]. An L-serine treatment precluded the development of neuropathy in the mouse model whereas a supplementation with alanine significantly aggravated the neuropathy in the HSAN1 animals. Thus, lowering 1-deoxySLs with L-serine supplementation offers a promising therapeutic target in HSAN1. Therefore, we were also interested to investigate this strategy in the diabetic neuropathy context.

Aim of the study:

In this work, we aimed to evaluate the potential of atypical sphingolipids as biomarkers for cardiometabolic diseases. In addition, we investigated whether a modulation of the plasma levels of these sphingolipids could be a therapeutic strategy in the context of diabetes and especially, the diabetic neuropathy.

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Chapter 1

Plasma Deoxysphingolipids: A Novel Class of Biomarkers for the Metabolic Syndrome?*†

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†The nomenclature used in this paper generally conforms to the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature [1] (see appendix).

Contribution statement

AO, MFR and DE did the lipid extraction and analysis, CHS, PR and HD were involved in study design, sample collection and patient characterisation, CPS, GLP, RP did the STZ rat experiments. AO did the statistical analysis, AvE contributed to study design, data interpretation and TH was involved in study design, data interpretation and wrote the manuscript. All authors had a substantial input in drafting the manuscript, critically appraising it and approved the final version to be published

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Abstract

Aims/hypothesis Sphingolipid synthesis is typically initiated by the conjugation of L-serine and palmitoyl-CoA, a reaction catalysed by the serine-palmitoyltransferase (SPT). SPT can also metabolise other acyl-CoAs (C_{12} to C_{18}) and other amino acids such as L-alanine and glycine, giving rise to a spectrum of atypical sphingolipids. Here, we aimed to identify changes in plasma levels of these atypical sphingolipids to explore their potential as biomarkers in the metabolic syndrome and diabetes.

Methods We compared the plasma profiles of ten sphingoid bases in healthy individuals with those of patients with the metabolic syndrome but not diabetes, and diabetic patients (n=25 per group). The results were verified in a streptozotocin (STZ) rat model. Univariate and multivariate statistical analyses were used.

Results Deoxysphingolipids (1-deoxySLs) were significantly elevated ($p=5\times10^{-6}$) in patients with the metabolic syndrome (0.11±0.04 µmol/l) compared with controls (0.06±0.02 µmol/l) but did not differ between the metabolic syndrome and diabetes groups. Levels of C_{16}-sphingosine-based sphingolipids were significantly lowered in diabetic patients but not in patients with the metabolic syndrome but without diabetes (p=0.008). Significantly elevated 1-deoxySL levels were also found in the plasma and liver of STZ rats. A principal component analysis revealed a similar or even closer association of 1-deoxySLs with diabetes and the metabolic syndrome in comparison with the established biomarkers.

Conclusions/interpretation We showed that 1-deoxySLs are significantly elevated in patients with type 2 diabetes mellitus and non-diabetic metabolic syndrome compared with healthy controls. They may, therefore, be useful novel biomarkers to improve risk prediction and therapy monitoring in these patients.

Abbreviations:

ALT	Alanine aminotransferase
1-deoxySA	Deoxysphinganine
1-deoxySO	Deoxysphingosine
1-deoxySLs	Deoxysphingolipids
DPN	Diabetic peripheral neuropathy
HSAN1	Hereditary sensory and autonomic neuropathy type 1
OPLS-DA	Orthogonal partial least square-discriminant analysis
PCA	Principal component analysis
ROC	Receiver operating characteristic
SA	Sphinganine
SO	Sphingosine
SPT	Serine palmitoyltransferase
STZ	Streptozotocin
VIP	Variable importance for the projection

Introduction

The pathogenesis of diabetes mellitus type 2 is characterised by the development of insulin resistance, frequently because of excess body fat, that is initially overcome by increased insulin secretion and—after several years—a subsequent decrease in the functional pancreatic beta cell mass that can no longer compensate for insulin resistance [2-4]. Insulin resistance or the compensating hyperinsulinaemia lead to the manifestation of a cluster of risk factors, including hyperglycaemia, hypertriacylglycerolaemia, low plasma levels of HDL-cholesterol and arterial hypertension, that has been termed the metabolic syndrome. Although much progress [5] has been made in the understanding of the mutual relationships between obesity, insulin resistance, type 2 diabetes and atherosclerosis, the complete picture remains elusive. In the last years the carbohydrate-centred view of the pathogenesis of diabetes has widened to include different classes of lipids [5-7] and inflammatory factors [8]. Mounting evidence suggests that sphingolipids play a role in the pathogenesis of insulin resistance and diabetes [9-11]. In particular, ceramides have been suggested to be important in the development of insulin resistance. For instance, inhibition of ceramide synthesis restored insulin sensitivity in palmitate-induced insulin resistance in mice [12].

Sphingolipids comprise a heterogeneous class of lipids that are derived from the aliphatic amino-alcohol sphingosine, which is commonly formed from the precursors L-serine and palmitoyl-CoA (electronic supplementary material [ESM] Fig. 1). This reaction is catalysed by the enzyme serine palmitoyltransferase (SPT). In addition to these substrates, SPT metabolises other acyl-CoAs with carbon chain lengths in the range C_{12} to C_{18} [13]. Moreover, SPT also has flexibility in the use of other amino acid substrates such as L-alanine and glycine [14, 15], which generate an atypical category of 1-deoxysphingoid bases. Whereas the conjugation of alanine forms deoxysphinganine (1-deoxySA), the use of glycine results in the formation of deoxymethylsphinganine. These two metabolites can be N-acylated to form deoxysphingolipids (1-deoxySLs), but cannot be further metabolised to complex sphingolipids because of the missing C_1-hydroxyl group [16]. Consequently, these metabolites cannot be degraded by the canonical sphingolipid degradation pathway, which requires the formation of sphingosine-1-phosphate as a catabolic intermediate. Whereas 1-deoxySLs occur ubiquitously at low levels in human plasma, levels are greatly increased in hereditary sensory and autonomic neuropathy type 1 (HSAN1; OMIM162400), an inherited sensory neuropathy caused by missense mutations in SPT [15].

Physiologically, de novo sphingolipid synthesis represents a metabolic cross point that interconnects lipid, amino acid and thereby indirectly also carbohydrate metabolism. Fluctuations in fatty acid and thereby acyl-CoA concentrations are reflected in altered carbon-chain compositions of the sphingoid bases [12, 17]. Another node connects serine and alanine metabolism to the sphingolipid metabolism. Serine is formed from 3-phosphoglycerate whereas alanine can be converted from and to pyruvate through alanine aminotransferase (ALT) in a reversible transaminase reaction. Hence, the precursors for both amino acids are generated during glycolysis and thereby provide a functional link between sphingolipid and carbohydrate metabolism. In this respect, it is noteworthy that alanine is the major gluconeogenic amino acid, with important roles in obesity and diabetes [18, 19].

In light of these metabolic connections and the increasing evidence that certain sphingolipid molecules have roles in the pathogenesis of insulin resistance and diabetes, we compared the plasma sphingoid base profiles of healthy humans with those of patients with the metabolic syndrome but not diabetes and of patients with type 2 diabetes mellitus. The observed differences were further confirmed in an animal model of diabetes.

Methods

Patients

A sex- and age-matched nested cohort of 25 patients with non-diabetic metabolic syndrome, 25 patients with the metabolic syndrome and type 2 diabetes mellitus, and 25 controls without diabetes or the metabolic syndrome was selected from a previously described study cohort [20] of the Vorarlberg Institute for Vascular Investigation and Treatment (VIVIT; Feldkirch, Austria). The samples were derived from a larger cohort of unselected white patients undergoing coronary angiography for the evaluation of suspected coronary artery disease. Only patients with negative angiographic results were enrolled in this study. The age range was 55 - 69 years. The angiographers were not aware of plasma sphingolipid levels. The study was approved by the Ethics Committee of the University of Innsbruck and all participants gave written informed consent.

According to National Cholesterol Education Program-Adult Treatment Panel III criteria, non-diabetic metabolic syndrome was diagnosed if three or more of the following criteria were fulfilled: waist circumference >102 cm in men or >88 cm in women; triacylglycerols ≥ 1.7 mmol/l (150 mg/dl); HDL-cholesterol < 1.0 mmol/l (40 mg/dl) in men or <1.3 mmol/l (50 mg/dl) in women; blood pressure ≥130 / ≥85 mmHg; and fasting glucose ≥6.1 mmol/l (110

mg/dl) but <7 mmol/l. Type 2 diabetes mellitus was diagnosed by either fasting glucose levels ≥ 7 mmol/l (126 mg/dl), or plasma glucose levels ≥ 11.1mmol/l (200 mg/dl) 2 h after an oral challenge with 75 g glucose, or previously diagnosed diabetes. Controls were defined by the absence of both non-diabetic metabolic syndrome and type 2 diabetes mellitus

Clinical chemistry

Venous blood samples were collected after an overnight fast of at least 12 h before angiography was performed, and laboratory measurements were performed from fresh serum samples. The serum levels of triacylglycerols, total cholesterol and HDL-cholesterol were determined by using enzymatic hydrolysis and precipitation techniques (Triglycerides *glycerol phosphate oxidase-p-aminophenazone* (GPO-PAP), cholesterol *oxidase phenol* 4-aminoantipyrine peroxidase (CHOD/PAP), QuantolipLDL, QuantolipHDL; Roche, Basel, Switzerland) on a Hitachi-Analyzer 717 or 911. Level of HbA_{1c} was determined by high-performance liquid chromatography (LC) on a Menarini-Arkray KDK HA 8140 (Arkray KDK, Kyoto, Japan). Clinical chemistry variables were measured on a Hitachi 717 or 911 system (Roche).

Animal model

Male Sprague Dawley rats (180-200 g, Charles River, Calco, Italy) were housed in pairs. Animal room temperature and relative humidity were set at 22±2°C and 55±10%, respectively. Artificial lighting provided a 12 h light–dark cycle (07:00–19:00 hours). The animals had free access to diet and water. Diabetes was induced in rats fasted overnight by a single i.p. injection of 60 mg/kg of streptozotocin (STZ) dissolved in sodium citrate buffer (pH 4.5). The control rats were injected with vehicle. Hyperglycaemia was confirmed by measuring glycosuria 72 h after STZ injection, using Keto-Diabur test 5000 strips (Roche Diagnostics, Spa, Italy). Only animals with glycosuria > 5% were classified as diabetic and included in the study. Body weight and blood glucose concentration, determined by tail bleeding using strips (Ascensia Elite; Bayer, Basel, Switzerland), were measured weekly. Immediately after killing the animals, liver and muscle (gastrocnemius) were carefully dissected out and immediately frozen in liquid N_2.

Quantification of sphingoid bases

The lipids were analysed as described before [15, 21]. Tissue samples were homogenised in lysis buffer (25mmol/l HEPES pH 8, 0.2% Triton X-100 (vol/vol)) using a Precellys 24 tissue

homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Briefly, 100µl plasma or tissue homogenate containing 80 µg extracted protein was added to 1 ml extraction buffer (2:1) methanol–KOH/chloroform and spiked with 200 pmol internal standard C_{20}-sphinganine (C_{20}SA; Avanti Polar Lipids, Alabaster, Alabama,USA).

The extracted dried lipids were acid hydrolysed using methanolic HCl (1 mol/l HCl/10 mol/l water in methanol) and heat (65°C, 16 h) to release the sphingoid base backbones. This was followed by a second extraction. LC/MS analysis was performed as described earlier [15]. Lipids were separated on a C_{18} column (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, Montluçon, France) and analysed by an MS detector (LCQ, Thermo, Reinach, BL, Switzerland). The samples were measured as singletons for each participant. Inter- and intra-assay CVs were between 5% and 20% for each sphingoid base.

The sphingoid bases analysed included C_{16}SO, C_{16}SA, C_{17}SO, C_{17}SA, C_{18}SO, C_{18}SA, C_{20}SO, C_{18}SA-diene, 1-deoxySA and 1-deoxySO.

Statistics

Mean comparison and receiver operating characteristic curve analyses The original dataset was imported into SPSS 16.0 (SPSS Switzerland, Zurich, Switzerland). As some of the variables measured did not follow normal distribution, even after transformations, non-parametric tests were used. The Kruskal–Wallis test was used to compare all three groups. The Mann–Whitney *U* test was used for comparisons between two groups, followed by the Bonferroni correction. Spearman correlations were calculated. Receiver operating characteristic (ROC) curve analysis was performed and the two-tailed asymptotic p value was calculated for the ROC AUCs.

Orthogonal partial least square-discriminant analysis Orthogonal partial least square-discriminant analysis (OPLS-DA) [22, 23] was used as a multivariate predictive and regression method, based on the concepts of principal component analysis (PCA). In PCA, a large number of correlated variables (e.g. clinical, clinical chemistry and sphingolipid variables) are summarised into a smaller number of uncorrelated ones called principal components. Thus, the correlation structures are preserved while reducing the number of variables. In contrast to PCA, which does not consider class membership, in OPLS-DA the group information is included (control, the non-diabetic metabolic syndrome and diabetes) as a *Y* variable. OPLS-DA therefore summarises the data into a predictive component, maximising the between-group variation and an orthogonal component(s) describing the

within-group variation. The predictive component describes the variations in *X* that correlate with *Y*. The orthogonal component describes the systematic variations within each group that do not correlate with *Y*, thereby removing the noise data from the predictive component.

For any modelling technique, model evaluation is necessary before any inferences are made. R^2Y reports the fit of the model to the original dataset and Q^2Y reports the predictive ability of the model calculated by cross validation. Cross validation is performed by dividing the whole dataset into multiple random groups and then predicting the class assignment of members of each group at a time.

The dataset was imported into SIMCA-P+ 12.0.1.0 (Umetrics, Umeå, Sweden) for the calculations. The groups (control, non-diabetic metabolic syndrome and diabetes) were assigned as classes. Variables that did not follow the normal distribution were log transformed before model fitting. As some variables did not follow the normal distribution after log transformation, models were fitted twice, once with the log-transformed variables and another time without any transformations. The model evaluation parameters and interpretations did not differ in both cases. Therefore, the values for all the variables without any transformations were used. As the values for the variables had different scales, the dataset was normalised in unit variance and centred around the mean. OPLS-DA models were fitted for the classes to get the highest R^2Y and Q^2Y values. Models were used to compare two groups only at a time (control vs non-diabetic metabolic syndrome and non-diabetic metabolic syndrome vs diabetes). We did not use three groups as a Y variable as this would have made the interpretations of the model results more complex.

As PCA-based methods are sensitive to outliers, Hotelling's T^2 and distance to the model, DmodX, were used to detect outliers. Hotelling's T^2 is a generalisation of the *t* distribution for the multivariate case and is usually visualised in score plots. Score plots show on the *x*-axis the scores of the predictive component describing the between-group variation and on the *y*-axis the scores of the orthogonal component describing the within-group variation. Tolerance ellipse is drawn in score plots outlining the 95% probability of the Hotelling's T^2 distribution. Scores for observations situated outside the tolerance ellipse can be considered outliers. Moreover, DModX shows the distance to the model in the *X* space in a way similar to the residuals in the linear regression models. A critical value D-Crit of 0.05 was set as a limit for outlier detection. After outliers were detected, models were fitted twice, excluding and including the outliers. In the current study, no difference was found in the model evaluation

parameters or any of the results upon inclusion or exclusion of the outliers. Therefore, models without outlier removal were used for the interpretation.

Cross validation was performed for each model with seven groups. The cross validation ANOVA was calculated along with misclassification tables and Fisher's probabilities.

Results

For this study, we analysed plasma samples from patients in three pre-defined subgroups, each consisting of 25 sex- and age-matched patients with either manifest type 2 diabetes mellitus, the metabolic syndrome but no manifest diabetes mellitus, or without diabetes mellitus or the metabolic syndrome (controls). The results are summarised in Table 1.

Sphingoid bases in plasma are usually *N*-acylated and conjugated with different headgroups, giving rise to a great variety of different sphingolipid species. To analyse the sphingoid base composition of these metabolites we subjected the extracted lipids to sequential acid and base hydrolysis to remove the *N*-acyl chain and head group. The resulting free sphingoid bases were analysed by LC/MS.

The most abundant sphingoid base in human plasma was C_{18}-sphingosine (C_{18}SO) followed by C_{18}SA-diene, C_{16}-sphingosine (C_{16}SO) and C_{17}-sphingosine (C_{17}SO). Generally, 1-deoxySLs were recovered as quantitatively minor fractions, representing 0.1-0.3% of the total sphingoid bases

Plasma concentrations of total 1-deoxySLs were significantly higher for the non-diabetic metabolic syndrome group (0.11±0.04 µmol/l) compared with controls (0.06±0.02 µmol/l) but did not differ between the metabolic syndrome and diabetic groups (Table 1). This elevation remained significant after the Bonferroni correction for multiple testing. In contrast, C_{16}SO levels were found to be significantly lower ($p= 0.008$) in diabetic patients (6.37± 2.82 µmol/l) in comparison with participants with non-diabetic metabolic syndrome (8.38±2.25 µmol/l). However, this decrease missed the significance limit after Bonferroni correction in which a significance level of 0.05 corresponds to a p value of 0.002. Concentrations of other sphingoid bases were not significantly different between the three groups.

Table 1 Baseline characteristics and results. Values are expressed as means±SD or percentages for scale or categorical variables, respectively, *p* values were calculated with Kruskal–Wallis test for all three groups, and with the Mann–Whitney *U* test for two group comparisons; after Bonferroni correction a *p* value of 0.002 corresponded to a significance level of 0.05. For the categorical variable, asymptotic two-sided *p* values for χ^2 are shown, AST, aspartate aminotransferase; C, controls; D, diabetic patient group; DBP, diastolic BP; MetS, metabolic syndrome group; SBP, systolic BP; TG, triacylglycerol; Wcf, waist circumference

Characteristic	**Control (n=25)**	**Metabolic syndrome (n=25)**	**Diabetes (n=25)**	**Kruskal–Wallis *p* value**	**Mann–Whitney *U p* value**	
					C vs MetS	**MetS vs D**
Age	61.19±4.28	62.13±5.02	61.56±4.64	0.771	0.50	0.567
Female (%)	40	40	40	1.000		
Smoking (%)	64	56	60	0.846		
Wcf (cm)	98.24±12.10	105.84±8.23	110.80±11.97	3.70E-04	0.004	0.051
BMI	28.19±4.49	30.13±3.01	32.21±4.83	0.006	0.05	0.09
Fasting glucose (mmol/l)	5.26±0.56	5.64±0.57	9.59±4.16	1.94E-09	0.03	4.29×10^{-7}
HbA_{1c} (%)	5.60±0.33	5.76±0.34	7.79±2.04	4.71E-10	0.17	9.52×10^{-8}
HbA_{1c} (mmol/l)	37.71	39.45	61.64			
Cholesterol (mmol/l)	5.26±1.24	4.99±1.30	4.98±1.44	0.777	0.49	0.861
LDL-cholesterol(mmol/l)	3.35±0.96	3.45±1.00	3.16±1.22	0.449	0.52	0.269
HDL-cholesterol(mmol/l)	1.80±0.69	1.15±0.21	1.31±0.32	1.60E-05	2.37E-06	0.132
TG (mmol/l)	1.10±0.37	2.51±0.83	2.21±1.47	1.96E-07	1.38E-08	0.067
SBP (mmHg)	126.80±12.47	133.84±15.23	135.52±15.92	0.081	0.12	0.44
DBP (mmHg)	78.80±8.50	83.92±9.41	82.24±8.99	0.161	0.06	0.565
AST	28.56±8.60	30.84±24.10	25.84±8.43	0.388	0.38	0.662
ALT	29.92±15.71	34.76±24.54	31.68±13.25	0.650	0.35	0.907
Creatinine	0.84±0.18	0.92±0.21	0.81±0.23	0.144	0.20	0.06
GFR	100.65±11.53	95.26±15.30	100.77±15.68	0.316	0.22	0.184
C_{16}SO (µmol/l)	8.31±4.49	8.38±2.25	6.37±2.82	0.041	0.55	0.008
C_{16}SA (µmol/l)	0.23±0.13	0.23±0.10	0.26±0.14	0.749	0.84	0.491
C_{17}SO (µmol/l)	4.66±2.42	4.02±1.10	3.71±1.48	0.387	0.64	0.29
C_{17}SA (µmol/l)	0.15±0.07	0.14±0.05	0.14±0.05	0.960	0.82	0.961
SO (µmol/l)	88.83±27.23	81.66±21.28	81.54±23.89	0.622	0.34	0.793
SA (µmol/l)	2.17±0.9	2.20±0.77	2.57±1.14	0.473	0.82	0.299
C_{20}SO (µmol/l)	0.20±0.08	0.19±0.06	0.19±0.05	0.727	0.46	0.839
1-deoxySO (µmol/l)	0.15±0.09	0.23±0.09	0.24±0.13	2.14E-04	3E-05	0.839
1-deoxySA (µmol/l)	0.06±0.02	0.11±0.04	0.12±0.05	3.92E-06	5E-06	0.808
C_{18}SA-diene (µmol/l)	20.79±8.17	18.27±5.74	16.42±6.80	0.148	0.36	0.273

Table 2 Results of STZ rat tissue and plasma analysis, Values are shown as mean ± SD, *p* values were calculated with Mann–Whitney *U* test: **p*<0.05; ****p*<0.001, C, controls; ND, not detected; STZ, STZ-treated rats

	Liver (pmol/100 µg protein)		Muscle (pmol/100 µg protein)		Plasma (µmol/l)	
Variable	C	STZ	C	STZ	C	STZ
Glucose (mmol/l)	-	-	-	-	8.8 ± 0.4	53.8 ± 3.3***
C_{16}SO	3.4 ± 0.8	3.1 ± 0.7	1.5 ± 0.23	1.4 ± 0.24	0.06 ± 0.02	0.09± 0.07
C_{17}SO	15.7 ± 3.3	13 ± 2.5	4.2 ± 0.44	5.0 ± 1.2	0.3 ± 0.1	0.4 ± 0.1
C_{18}SA-diene	64.1 ± 10.6	78.1 ± 26.1	11.2 ± 2.3	21.5 ± 10.3*	2.6 ± 0.4	5.0 ± 0.9*
SO	1130 ± 189	1127 ± 252	180 ± 25.4	262 ± 106*	24.3 ± 7.6	29.1 ± 4.8
SA	40.3 ± 9.4	66.4 ± 29*	9.1 ± 1.8	8.1 ± 2.6	1.0 ± 0.5	1.6 ± 0.6*
C_{20}SO	10.8 ± 4.4	11.4 ± 2	6.5 ± 0.6	6.7 ± 1.6	0.1 ± 0.08	0.15 ± 0.9
1-deoxySA	0.2 ± 0.08	0.4 ± 0.12***	ND	ND	0.01 ± 0.003	0.12 ± 0.9***
1-deoxySO	ND	ND	ND	ND	ND	ND

A correlation matrix of all variables (clinical data, clinical chemistry measurements along with sphingoid base backbone measurements) showed a significant correlation of the serine-based sphingolipids (C_{16}, C_{17} or C_{18}SA or SO and C_{18}SA-diene) with LDL-cholesterol and total cholesterol but correlated less strongly with HDL-cholesterol. In contrast, the alanine-based 1-deoxySLs showed a strong correlation with the variables related to the metabolic syndrome, such as waist circumference and triacylglycerols (ESM Fig. 2). Therefore, we were interested to see whether the increased 1-deoxySL levels are associated with hyperglycaemia per se or rather with insulin resistance. To address this issue we analysed plasma, liver and muscle tissue samples from STZ-treated rats (Table 2). Although beta cell failure and not insulin resistance is the basis of the STZ rat model, we found significantly elevated 1-deoxySL levels in plasma and liver from STZ rats (Table 2, Fig. 1). No 1-deoxySLs were detected in muscle tissue. This suggests that plasma 1-deoxySLs are primarily elevated in association with hyperglycaemia and are probably of hepatic origin.

An OPLS-DA was used to estimate the importance of the individual variables as discriminating biomarkers. Both models (control vs non-diabetic metabolic syndrome and non-diabetic metabolic syndrome vs diabetes) showed good fit and predictive power. Therefore, they are valid for discrimination (ESM Table 1).

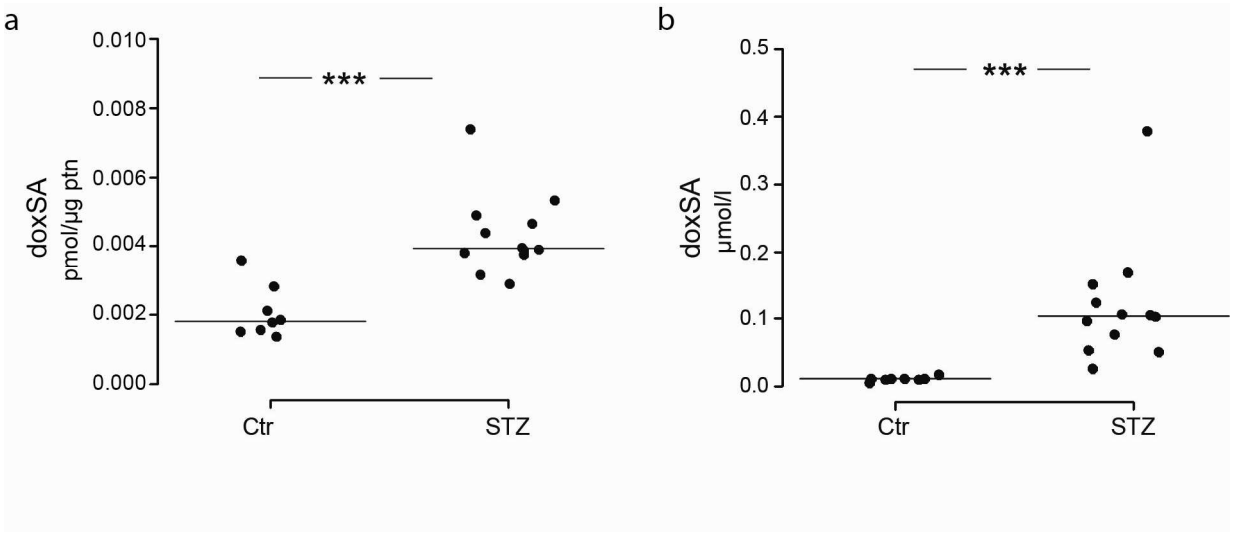

Fig. 1 1-deoxySA levels are significantly (p<0.001) elevated in liver tissue (**a**) and plasma (**b**) of STZ rats (STZ) compared with controls (C)

In score plots (Fig. 2a,b), clustering into separate groups was preserved after reducing the whole dataset into a single predictive component (shown on the x-axis) and an orthogonal one (shown on the y-axis). Loading plots (Fig. 2c,d) show the weights of each of the original variables in the model and hence their individual contributions to the disease state. In the control vs metabolic syndrome loading plot (Fig. 2c), triacylglycerols, 1-deoxySA, 1-deoxySO, and diastolic and systolic blood pressure contributed to the metabolic syndrome state model (95% CI>0) whereas elevated HDL-cholesterol contributed to the control state model (95%CI<0). This confirms the biological and clinical validity of the model, as elevated triacylglycerols, low HDL-cholesterol and hypertension are key features of the metabolic syndrome. 1-deoxySA and 1-deoxySO were identified as the second and fourth most important contributors to the metabolic syndrome model, just after triacylglycerols and HDL-cholesterol, respectively, but above glucose, waist circumference and systolic blood pressure. In the metabolic syndrome vs diabetes model (Fig. 2d), we found elevated fasting glucose and HbA_{1c} concentrations, together with low $C_{16}SO$ and creatinine levels, to be the contributors to the diabetes state model.

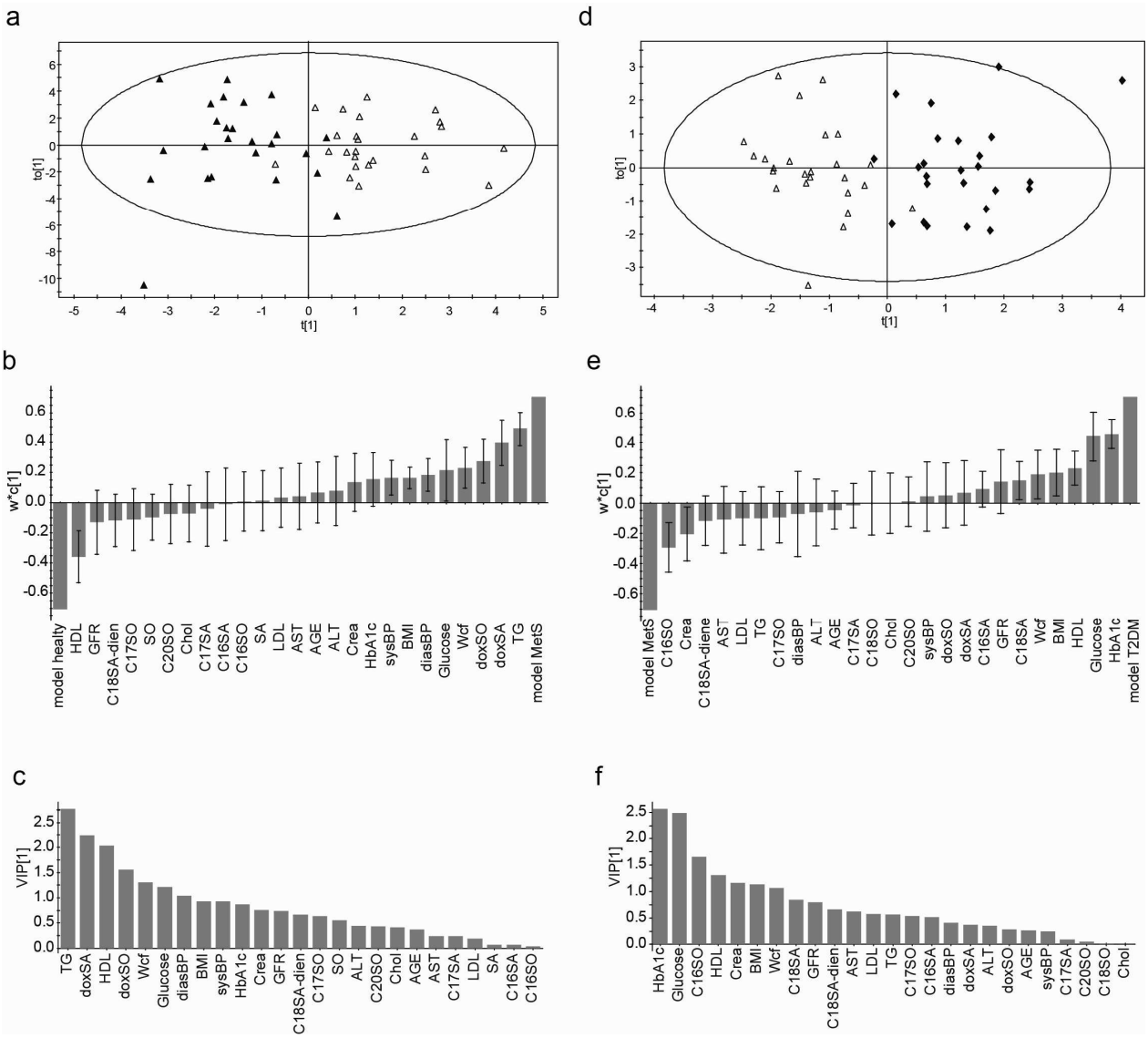

Fig. 2 **a**,**b** Score plots of OPLS-DA models: (**a**) control vs non-diabetic metabolic syndrome; (**b**) non-diabetic metabolic syndrome vs type 2 diabetes. Individual observations are shown as: black triangles, controls; white triangles, the metabolic syndrome; black rhombus, type 2 diabetes. On the *x*-axis, the scores for each individual in the predictive principal component (t[1]) are shown, while the *y*-axis shows the scores for each individual in the orthogonal component (to[1]). Thus, variations on the *x*-axis reflect the between-group separation while variations on the y-axis show the within-group variations (noise). The tolerance ellipse corresponds to 95% of the Hotelling's T^2 multivariate distribution. **c**,**d** Loading column plots of OPLS-DA models control vs the metabolic syndrome (**c**) and the metabolic syndrome vs diabetes (**d**). The variables are shown on the *x*-axis, while the loading coefficients (weights) are shown on the y-axis.The weights represent the contribution of each variable to the model component scores. Variables with larger weights contribute more to the model. Error bars represent 95% CIs for calculated weights. **e**,**f** VIP plot for OPLS-DA models: control vs the metabolic syndrome (**e**) and the metabolic syndrome vs diabetes (**f**). The variables are shown on the *x*-axis, while VIP coefficients are shown on the *y*-axis. The VIP coefficients plot shows the summation of all the weights for each *X* variable to predict *Y* and hence denoting the importance of each *X* variable. Chol, cholesterol; Crea, creatinine; DBP, diastolic BP; HDL, HDL-cholesterol; LDL, LDL-cholesterol; SBP, systolic BP; T2DM, type 2 diabetes mellitus; TG, triacylglycerol; Wcf, waist circumference;

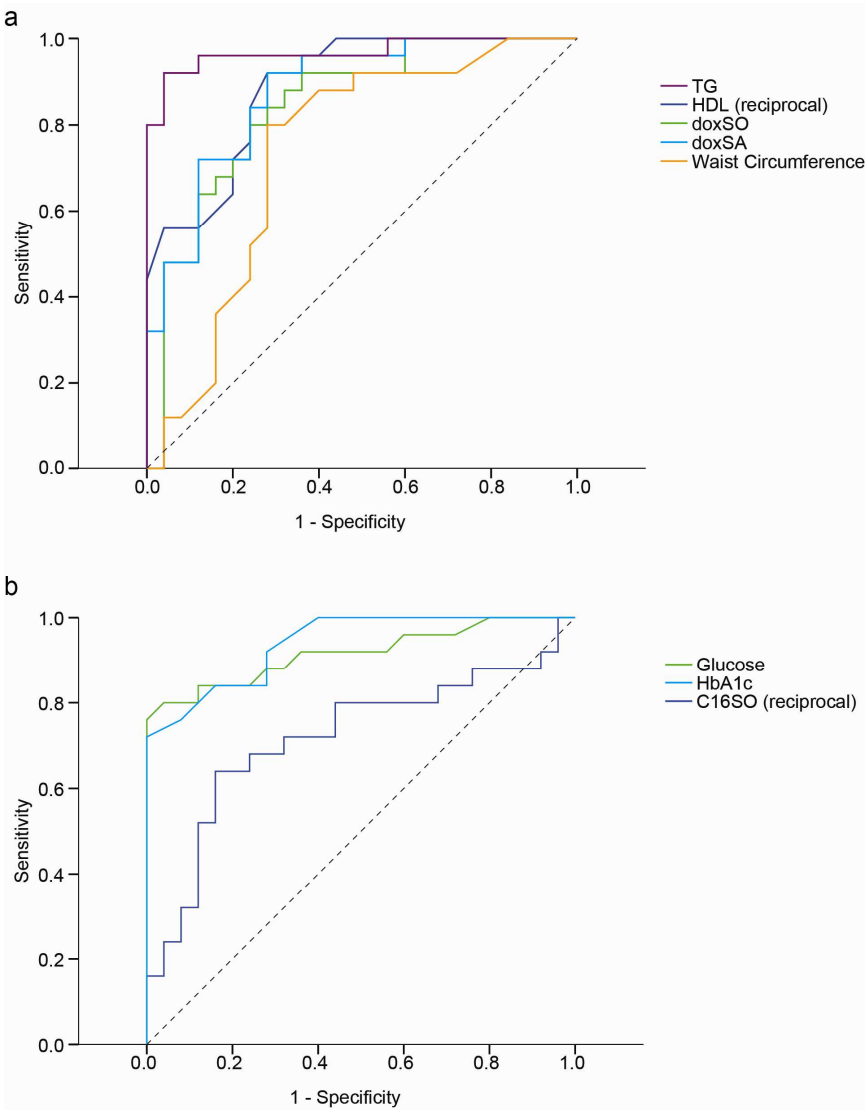

Fig. 3 ROC curves. (**a)** ROC curves for control vs metabolic syndrome showing triacylglycerols and HDL-cholesterol as predictors of the metabolic syndrome with AUCs of 0.968 (p<0.001) and 0.111 (corresponding to 0.889), respectively. The 1-deoxySLs show comparable AUCs, with 0.875 for 1-deoxySA and 0.842 for 1-deoxySO. Purple, triacylglycerol; dark blue, HDL-cholesterol (reciprocal); green, 1-deoxySO; light blue, 1-deoxySA; orange, waist circumference. (**b)** ROC curves for potential biomarkers in metabolic syndrome vs diabetes. HbA_{1c} and glucose show AUCs of 0.939 and 0.917 for type 2 diabetes, whereas $C_{16}SO$ shows a significantly lower AUC of 0.282 (corresponding to 0.718), denoting its decrement in association with diabetes. Green, glucose; light blue, HbA_{1c}; dark blue, $C_{16}SO$ (reciprocal)

Variable importance for the projection (VIP) plots (Fig. 2e,f) show the contribution of each variable to the variation in both the *X* space and the *Y* space (and hence their correlations with other variables and the control or disease state). A coefficient value >1 signifies that the variable is important. For the control vs metabolic syndrome model (Fig. 2e), the VIP plot showed the highest VIP coefficients for triacylglycerols, 1-deoxySA, HDL-cholesterol and 1-deoxySO. A slight importance (VIP coefficient slightly >1) was noticed for diastolic pressure and fasting glucose. For the metabolic syndrome vs type 2 diabetes mellitus model (Fig. 2f) a

significant importance of HbA_{1c}, glucose and $C_{16}SO$ was seen. VIP coefficients were also >1 for BMI, HDL-cholesterol and creatinine.

In summary, the OPLS-DA analysis revealed that triacylglycerols, 1-deoxySA, 1-deoxySO and HDL-cholesterol are the best explanatory variables for the non-diabetic metabolic syndrome, while differences in HbA_{1c}, glucose and $C_{16}SO$ were mostly related to the diabetes.

The diagnostic potential of these markers was analysed in an ROC curve analysis (Fig. 3a,b). For the diagnosis of the metabolic syndrome, 1-deoxySA and 1-deoxySO (Fig. 3a) had AUCs of 0.875 and 0.842, respectively ($p<0.001$). Moreover, $C_{16}SO$ (Fig. 3b) showed an AUC of 0.282 (corresponding to 0.718; $p<0.01$).

Discussion

In this study, we compared the plasma sphingoid base compositions of healthy individuals with those of individuals with non-diabetic metabolic syndrome and diabetic patients. We found 1-deoxySLs to be significantly elevated in the plasma of patients with either non-diabetic metabolic syndrome or type 2 diabetes mellitus compared with controls but not different between participants with non-diabetic metabolic syndrome and diabetic patients. In contrast, $C_{16}SO$ levels were lower in diabetic patients but did not differ between controls and metabolic syndrome patients. Other sphingoid base metabolites were not different between the groups (Table 1).

These results indicate that the metabolic changes in the metabolic syndrome and type 2 diabetes mellitus are specifically associated with alterations in some, but not all, sphingoid base species. Elevated 1-deoxySL levels were also confirmed in the plasma and liver tissue of a model of type 1 diabetes (STZ rats), indicating that the observed increase in 1-deoxySLs is independent of the type of diabetes.

Sphingolipid metabolism can be considered as a metabolic cross point that interconnects fatty acid (acyl-CoA) and amino acid (serine and alanine) metabolism. Serine and alanine formation is thereby linked to glycolysis, which forms their precursors, 3-phosphoglycerate and pyruvate, respectively.

The metabolic syndrome and type 2 diabetes mellitus are associated with the clustering of several risk factors. To delineate the relative contribution of each variable we used a supervised learning approach (OPLS-DA) [24, 25]. This analysis showed that 1-deoxySL levels have, in addition to triacylglycerols and HDL-cholesterol, the highest explanatory

power for the metabolic syndrome. In this context it has to be considered that the metabolic syndrome and type 2 diabetes mellitus are clinically not two completely separate entities. Most diabetic patients also fulfil the criteria for the metabolic syndrome which, by itself, is associated with insulin resistance. In fact, most diabetic patients have presented for many years with the metabolic syndrome before hyperglycaemia has manifest. The observation that 1-deoxySL levels are not different between non-diabetic metabolic syndrome patients and diabetic patients suggests that the 1-deoxySLs are already formed in the prediabetic state in which insulin insensitivity is still compensated by increased insulin production and hyperinsulinaemia.

In contrast, C_{16}SO together with glucose and HbA_{1c} were the strongest contributors in the diabetes model. C_{16}SO is generated by the use of myristoyl-CoA instead of palmitoyl CoA—a reaction that is primarily catalysed by the SPT long-chain base subunit 3 (SPTLC3) [13].

It should be noted at this point that we also found significant levels of C_{17}SO in the plasma samples analysed. C_{17}SO is considered to be an ‘unnatural’ sphingoid base and therefore is sometimes used as an internal normalisation standard in lipidomics. However, the identity of C_{17}SO in human plasma was validated by comparison with a commercial synthetic C_{17}SO standard and also reported earlier by Quehenberger et al. [26]. This indicates that C_{17}SO has to be used with caution as an internal standard for plasma samples.

Currently, we do not fully understand why plasma 1-deoxySL levels are increased in the metabolic syndrome and diabetes. That 1-deoxySLs are almost exclusively present in VLDL and LDL but not HDL indicates that the 1-deoxySLs in plasma are primarily of hepatic origin [27]. This view is supported by the observation that 1-deoxySL levels were elevated in liver and plasma of STZ rats but were not present in skeletal muscle. A possible explanation for the increased 1-deoxySL formation might be an increased hepatic availability of alanine. Recent reports show that lifestyle modifications in the metabolic syndrome are associated with significant changes in the plasma amino acid profiles [28]. In obese individuals increases in skeletal muscle output of alanine and hepatic uptake of alanine were seen while serine levels were not changed [18]. Furthermore, hepatic glucose uptake is primarily mediated by GLUT2 and hence is insulin independent. Hyperglycaemic conditions are therefore associated with elevated hepatic glucose levels and an increased glycolytic flux that increases the formation of pyruvate and its anaerobic conversion to either lactate or alanine. Elevated glucose levels could hence increase hepatic alanine levels and thereby 1-deoxySL generation. This model implies that 1-deoxySL levels are elevated independent of the type of diabetes, which is

supported by the observation that 1-deoxySL levels were also found to be elevated in the plasma and liver of an animal model of type 1 diabetes (STZ rats). In both conditions even short-term and transient fluctuations in plasma glucose levels might be integrated over time leading to increased formation of 1-deoxySLs that, by themselves, might have a slow turnover as they are not degraded by the canonical sphingolipid catabolism.

Another interesting aspect is whether increased plasma 1-deoxySL levels as well as decreased plasma C_{16}SO levels contribute to the pathogenesis of diabetes and its complications. Genome-wide association studies have shown a strong association of genetic *SPTLC3* variants with alterations in lipid metabolism [29] and increased risk for myocardial infarction [30]. Therefore, lower C_{16}SO levels might be directly or indirectly related to the increased risk of diabetic patients for developing cardiovascular complications.

Previously, we showed that the increased formation of 1-deoxySLs is the pathological cause of the inherited neuropathy HSAN1 [15, 31]. Clinically, HSAN1 closely resembles the diabetic peripheral neuropathy (DPN) that occurs in about 60% of diabetic patients. Both conditions have late onset, slow progression and length-dependent axonopathy. All peripheral nerves are affected, including pain fibre, motor neurons and autonomic nerves. The degeneration of small sensory fibres results in the loss of pain sensation, which in turn leads to painless injuries. Furthermore, HSAN1 and DPN are both associated with skin ulcers, which is not a common feature in other peripheral neuropathies. Considering the neurotoxic properties of 1-deoxySLs, it might therefore be conceivable that higher levels of 1-deoxySLs are not only interesting from the biomarker perspective but also because 1-deoxySLs are potential pathogenic agents in the DPN. Interestingly, it has been shown recently that serum triacylglycerols correlate with the progression of the diabetic neuropathy [32]. In parallel we observed a strong correlation of the 1-deoxySLs and triacylglycerol levels (ESM Fig. 2).

Taken together our results suggest that 1-deoxySLs are relevant biomarkers for both the metabolic syndrome and type 2 diabetes mellitus, whereas C_{16}SO bases seem to be specifically lowered in diabetes, possibly reflecting the risk for developing cardiovascular complications. Therefore, a combination of these markers might help to improve risk prediction and therapy monitoring in diabetic patients. This could be especially relevant in the transition from the prediabetic to the diabetic state. A limitation of this study is the rather small group size. However, for a pilot biomarker study it is valid to use small numbers as long as type I and type II errors are considered carefully. We showed p values in the order of 1×10^{-5} after the most conservative correction for 1-deoxySA and 1-deoxySO, which support

rejection of the null hypothesis, that there is no difference in their plasma levels between the different groups. However, larger and ideally prospective follow-up studies are needed to further validate the potential of these metabolites and to explore their diagnostic and prognostic value. Finally, a more detailed knowledge of the physiological and pathophysiological properties of these metabolites is important to better understand the interplay between sphingolipids, carbohydrate metabolism, insulin resistance and diabetes.

Acknowledgements

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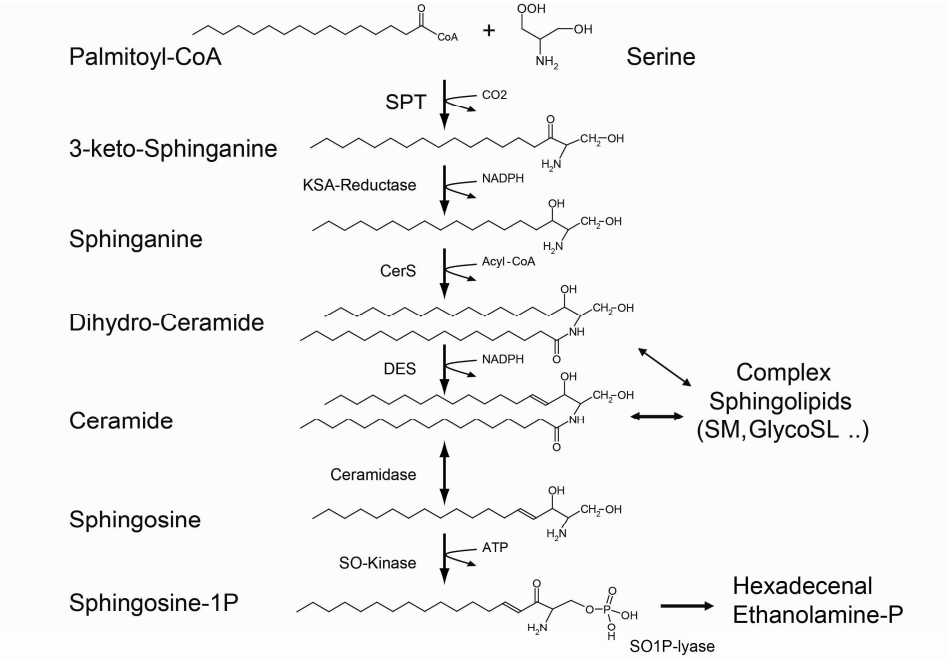
Duality of interest the authors declare that there is no duality of interest associated with this manuscript.

Appendix

Chemical nomenclature

C_{16}SA	(2*S*,3*R*,4*E*)-2-Aminohexadecan-1,3-diol
C_{17}SA	(2*S*,3*R*,4*E*)-2-Aminoheptadecan-1,3-diol
C_{18}SA	(2*S*,3*R*,4*E*)-2-Aminooctadecan-1,3-diol
C_{20}SA	(2*S*,3R,4*E*)-2-Aminoicosan-1,3-diol
C_{16}SO	(2*S*,3*R*,4*E*)-2-Amino-4-hexadecen-1,3-diol
C_{17}SO	(2*S*,3*R*,4*E*)-2-Amino-4-heptadecen-1,3-diol
C_{18}SO	(2*S*,3*R*,4*E*)-2-Amino-4-octadecen-1,3-diol
C_{20}SO	(2*S*,3*R*,4*E*)-2-Amino-4-icosen-1,3-diol

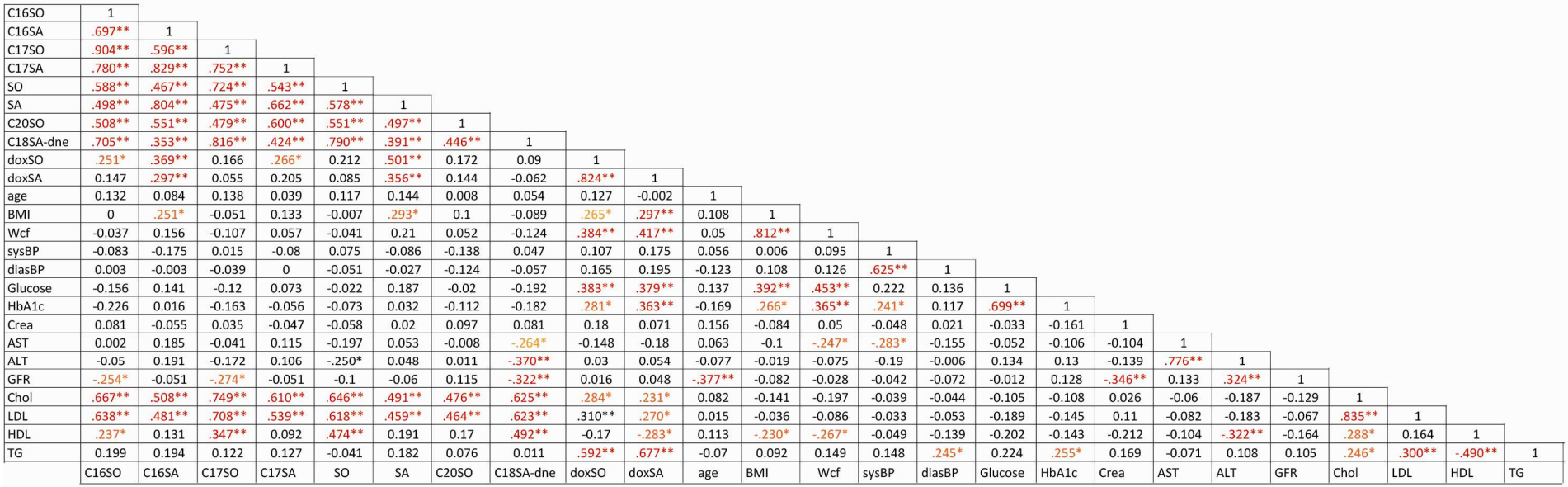
Supplementary material



supp. Figure 1 Sphingolipid de-novo synthesis pathway

Model	R^2X	R^2Y	Q^2Y	CV-ANOVA F value	CV-ANOVA P value	Correct classification	Fisher`s propability
Control vs Metabolic Syndrome	0.34	0.64	0.52	12.02	1.0e-6	94%	6.2e-11
Metabolic Syndrome vs Diabetes	0.38	0.66	0.33	3.32	0.0094	92%	5.3e-10

suppl. Table 1 OPLS-DA model evaluation. R^2X, R^2Y and Q^2Y represent the amount of explained X-variation, Y variation and predicted Y-variation, respectively. Cross validation (CV) is evaluated by CV-ANOVA p value which represents the significance of the predicted Y variation for that F value. Misclassification table results are shown as percentage of correct classification of the entire group along with Fisher's probability. Both models explain to a good extent the variations in Y, show good cross validation significance and can be used for prediction.



	C16SO	C16SA	C17SO	C17SA	SO	SA	C20SO	C18SA-dne	doxSO	doxSA	age	BMI	Wcf	sysBP	diasBP	Glucose	HbA1c	Crea	AST	ALT	GFR	Chol	LDL	HDL	TG
C16SO	1																								
C16SA	.697**	1																							
C17SO	.904**	.596**	1																						
C17SA	.780**	.829**	.752**	1																					
SO	.588**	.467**	.724**	.543**	1																				
SA	.498**	.804**	.475**	.662**	.578**	1																			
C20SO	.508**	.551**	.479**	.600**	.551**	.497**	1																		
C18SA-dne	.705**	.353**	.816**	.424**	.790**	.391**	.446**	1																	
doxSO	.251*	.369**	0.166	.266*	0.212	.501**	0.172	0.09	1																
doxSA	0.147	.297**	0.055	0.205	0.085	.356**	0.144	-0.062	.824**	1															
age	0.132	0.084	0.138	0.039	0.117	0.144	0.008	0.054	0.127	-0.002	1														
BMI	0	.251*	-0.051	0.133	-0.007	.293*	0.1	-0.089	.265*	.297**	0.108	1													
Wcf	-0.037	0.156	-0.107	0.057	-0.041	0.21	0.052	-0.124	.384**	.417**	0.05	.812**	1												
sysBP	-0.083	-0.175	0.015	-0.08	0.075	-0.086	-0.138	0.047	0.107	0.175	0.056	0.006	0.095	1											
diasBP	0.003	-0.003	-0.039	0	-0.051	-0.027	-0.124	-0.057	0.165	0.195	-0.123	0.108	0.126	.625**	1										
Glucose	-0.156	0.141	-0.12	0.073	-0.022	0.187	-0.02	-0.192	.383**	.379**	0.137	.392**	.453**	0.222	0.136	1									
HbA1c	-0.226	0.016	-0.163	-0.056	-0.073	0.032	-0.112	-0.182	.281*	.363**	-0.169	.266*	.365**	.241*	0.117	.699**	1								
Crea	0.081	-0.055	0.035	-0.047	-0.058	0.02	0.097	0.081	0.18	0.071	0.156	-0.084	0.05	-0.048	0.021	-0.033	-0.161	1							
AST	0.002	0.185	-0.041	0.115	-0.197	0.053	-0.008	-.264*	-0.148	-0.18	0.063	-0.1	-.247*	-.283*	-0.155	-0.052	-0.106	-0.104	1						
ALT	-0.05	0.191	-0.172	0.106	-.250*	0.048	0.011	-.370**	0.03	0.054	-0.077	-0.019	-0.075	-0.19	-0.006	0.134	0.13	-0.139	.776**	1					
GFR	-.254*	-0.051	-.274*	-0.051	-0.1	-0.06	0.115	-.322**	0.016	0.048	-.377**	-0.082	-0.028	-0.042	-0.072	-0.012	0.128	-.346**	0.133	.324**	1				
Chol	.667**	.508**	.749**	.610**	.646**	.491**	.476**	.625**	.284*	.231*	0.082	-0.141	-0.197	-0.039	-0.044	-0.105	-0.108	0.026	-0.06	-0.187	-0.129	1			
LDL	.638**	.481**	.708**	.539**	.618**	.459**	.464**	.623**	.310**	.270*	0.015	-0.036	-0.086	-0.033	-0.053	-0.189	-0.145	0.11	-0.082	-0.183	-0.067	.835**	1		
HDL	.237*	0.131	.347**	0.092	.474**	0.191	0.17	.492**	-0.17	-.283*	0.113	-.230*	-.267*	-0.049	-0.139	-0.202	-0.143	-0.212	-0.104	-.322**	-0.164	.288*	0.164	1	
TG	0.199	0.194	0.122	0.127	-0.041	0.182	0.076	0.011	.592**	.677**	-0.07	0.092	0.149	0.148	.245*	0.224	.255*	0.169	-0.071	0.108	0.105	.246*	.300**	-.490**	1

suppl. Figure 2 Spearman correlation matrix (2-tailed) of the analysed variables. Significant correlations are highlighted, *p< 0.05, **p<0.01

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Chapter 2

Plasma Sphingolipid Profiling Reveals Novel Distinct Biomarkers for Predicting Cardiovascular Disease and Type 2 Diabetes Mellitus*

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Contribution statement

AO did the lipid extraction, mass spectrometric analysis, the statistical analysis and wrote the manuscript. CHS and HD were involved in study design, sample collection and patient characterization. AvE contributed to study design, data interpretation and critically revised the manuscript. TH was involved in study design, data interpretation and supervision and critically revised the manuscript.

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Abstract:

Background:

Cardio-metabolic diseases remain to be a major cause of mortality and morbidity, despite the progress in risk prediction and stratification. Besides the known risk factors, sphingolipids are emerging as novel players in the pathogenesis of atherosclerosis and metabolic diseases. Sphingolpid de-novo biosynthesis is typically initiated by the condensation of palmitoyl-CoA and serine, a reaction catalyzed by the serine palmitoyltransferase (SPT). Besides these canonical substrates, SPT can also metabolize other acyl-CoAs and other amino acids, thereby generating a great variety of atypical sphingoid bases. In this study, we investigated the predictive potential of these atypical sphingoid bases in cardiovascular disease and type 2 diabetes mellitus (T2DM).

Methods:

Subjects enrolled in this study were selected from patients referred to angiography for the evaluation of established or suspected coronary artery disease between September 1999 and October 2000 (VIVIT study). They were followed up for cardiovascular events and the development of T2DM over a period of 8 years (median 7.7 years, range [16 days- 8.8 years]). In a cohort (n = 349), the sphingoid base profile of plasma sphingolipids was analyzed. Total sphingolipids were extracted, hydrolyzed and the sphingoid base backbones determined by LC/MS.

Results:

Confirming our earlier results, we observed that plasma 1-deoxysphingolipids (1-deoxySLs) were significantly elevated in patients with the metabolic syndrome and T2DM. In contrast, plasma C_{18}SO, C_{18}SAdiene and C_{18}SA- based sphingolipids were significantly lowered in patients with coronary artery disease. In the prospective analysis, baseline 1-deoxySLs were identified as independent predictors for the development of T2DM (OR = 2.05, CI 95% [1.15-3.64]) even after adjustment for HbA1c and the presence of metabolic syndrome. ROC curve analysis, discriminating the group which developed T2DM, showed a significantly higher AUC of the combined variables (1-deoxySO, HbA1c and metabolic syndrome) than for each of the covariates alone (AUC = 0.77, p = 0.01).

Additionally, baseline C_{20}SO sphingoid base backbones were identified as independent predictors for the risk to develop cardiovascular events (HR = 1.31, CI 95% [1.1-1.56]) even

after the adjustment for traditional risk factors including the degree of coronary artery stenosis at baseline. Interestingly, cross sectional plasma C20-based sphingolipids were not significantly different between CAD patients and controls.

Conclusion:

We show that 1-dexoySLs are promising predictive biomarkers for the development of T2DM whereas C20SO are prognostic biomarkers for the prediction of cardiovascular events. They might, therefore, provide novel tools for the risk prediction of cardio-metabolic diseases.

Abbreviations:

1-deoxySA	1-deoxysphinganine
1-deoxySO	1-deoxysphingosine
1-deoxySLs	1-deoxysphingolipids
AUC	Area under the curve
$C_{16\text{-}19}$	Carbon chain length (16 carbons -19 carbons)
CAD	Coronary artery disease
HR	Hazards ratio
HSAN1	Hereditary sensory and autonomic neuropathy type I
OR	Odds ratio
ROC	Receiver operating characteristic
SA	Sphinganine
SO	Sphingosine
SPT	Serine palmitoyltransferase
T2DM	Type 2 diabetes mellitus

Introduction:

Cardiovascular diseases and the related metabolic disorders remain a major cause of mortality and morbidity worldwide, despite the advances in risk prediction, diagnosis and management. Although traditional risk factors such as smoking, diabetes, dyslipidemia and hypertension help to identify patients with increased risk for cardiovascular events [1], a significant group of coronary heart disease patients (15-20%) cannot be identified based on these factors [1]. Similarly, impaired fasting glucose and impaired glucose tolerance fail to predict almost 50% of the patients who will develop T2DM [2, 3]. This fact has fueled an expanding research pursuit [4, 5] to identify risk factors and novel biomarkers to reclassify patients into better predictive risk categories. However, most of these novel biomarkers fail to improve the predictive ability of the current risk algorithms. It was, therefore, suggested that factors which are not apparently involved in the disease pathogenesis could serve better as predictive biomarkers [6]. With the progress in mass spectrometry and nuclear magnetic resonance, many blind spots in the human plasma metabolome and lipidome are becoming visible and quantifiable. This might lead to the discovery of novel biomarkers that add further information to the current predictive algorithms. Hereby a variety of metabolites such as acylcarnitines, amino acids, phospholipids and also sphingolipids have been suggested as promising biomarkers for the prediction of T2DM [7-9] and cardiovascular diseases [10].

Sphingolipids comprise a heterogeneous class of lipids involving ceramides, sphingomyelins, glycosphingolipids and free sphingoid bases. They are typically formed from the precursors L-serine and palmitoyl-CoA, in a reaction catalyzed by the enzyme serine palmitoyltransferase (SPT). The product of SPT is rapidly reduced to sphinganine, (C_{18}SA) which can be further metabolized to form ceramides, sphingomyelin and gylcosphingolipids. These metabolites are degraded to form sphingosine (C_{18}SO) which can be phosphorylated to form sphingosine 1-phosphate, irreversibly degraded or recycled into ceramides. C_{18}SA and C_{18}SO are the typical sphingoid bases or sphingoid base backbones found in sphingolipids. SPT is primarily composed of the three subunits SPTLC1, SPTLC2 and SPTLC3 which bind further proteins that are involved in regulating substrate specificity and enzyme activity [11-13]. Besides the canonical substrates L-serine and palmitoyl-CoA, SPT is able to metabolize other amino acids such as L-alanine and glycine and other acyl-CoAs with a carbon chain length in the range of C_{12} to C_{18} as alternative substrates. The reaction with alanine generates an atypical category of 1-deoxysphingolipids (1-deoxy-SL) which lack the C_1 hydroxyl group of the typical sphingolipids. On other hand, the use of stearoly-CoA instead of palmitoyl-CoA

results in sphingolipids with a C20 sphingoid base backbone (C_{20} sphingolipids). The use of different acyl-CoAs therefore leads to a considerable diversity in the sphingoid base backbones. In this respect, the SPT reaction provides thereby also a metabolic connection between amino acid (serine, alanine and glycine) and fatty acid metabolisms.

Sphingolipids were shown to be involved in the development of atherosclerosis [14] and metabolic diseases [15]. Elevated plasma sphingomyelin levels were found in patients with coronary artery disease [16] and have been suggested to be associated with subclinical atherosclerosis [17], although this association was not anymore significant after adjustment for the traditional risk factors. Moreover, plasma sphingomyelin was reported as an independent predictor of myocardial infarction (MI) and cardiovascular death in acute coronary syndrome patients [18] but was not associated with future incident MI or cardiovascular death in patients with stable angina [18] or in subjects free of clinical cardiovascular diseases at baseline [19]. Myriocin, a potent SPT inhibitor, has been suggested to help against atherosclerosis by inhibiting sphingolipid biosynthesis in animals [20-22]. The oral administration of myriocin to ApoE-KO mice on western diet led to decreased plasma sphingomyelin, cholesterol, triglycerides and increased HDL cholesterol which resulted in a reduced formation of atherosclerotic lesions [23]. These lipid-lowering effects were shown to be associated with a decreased expression of SREBP-1 and an increased LCAT activity [20]. In contrast, the intra-peritoneal injection of myriocin, had similar effects on sphingomyelin and atherosclerosis in ApoE-KO (on high fat diet), but did not influence total cholesterol or triglycerides levels [21, 24]. It has also been suggested that myriocin could even promote the regression of atherosclerosis [22]. Moreover, myriocin was shown to improve insulin resistance and preclude the development of T2DM in animal models [25]. Ceramides were shown to counteract the insulin action on glucose uptake and glycogen synthesis by inhibiting protein kinase B/Akt through different mechanisms [26]. Recently, it has been suggested that many of the beneficial effects of adiponectin on insulin sensitivity are linked to its lowering effect of intracellular ceramide levels [27]. Plasma ceramides were found elevated in patients with T2DM [28] and ceramide levels were shown to correlate with the level of insulin resistance in these patients. In the plasma of diabetic monkeys, sphingomyelin and gylcosphingolipids were decreased [29]. In our own work, we showed previously that 1-deoxySLs were significantly elevated in metabolic syndrome [30] and T2DM [31].

In light of these findings and the fact that SPT forms a metabolic cross-road between amino acid and acyl-CoA metabolism, we aimed to evaluate the predictive role of plasma

sphingolipids in cardiovascular disease and T2DM, with a particular focus on the various sphingoid base backbones.

Methods:

Patients and study design:

For this study, a cohort of 349 patients were selected from the VIVIT study [32] based on the availability of follow-up data for T2DM and cardiovascular events. The analysed individuals were enrolled in the VIVIT study between September 1999 and October 2000. At baseline all subjects underwent coronary angiography to evaluate a suspected or established coronary artery disease. Anthropometric data were collected, clinical chemistry lab parameters were measured and coronary angiography (Judkin`s technique) was performed on all the study participants at baseline.

Metabolic syndrome was defined according to the criteria of the National Cholesterol Education Program-Adult Treatment Panel III criteria, if three or more of the following criteria were met: waist circumference > 102 cm in men or > 88 cm in women; triglycerides ≥ 1.7 mmol/l (150 mg/dl); HDL-cholesterol < 1.0 mmol/l (40 mg/dl) in men or < 1.3 mmol/l (50 mg/dl) in women; blood pressure ≥ 130 / ≥ 85 mmHg; and fasting glucose ≥ 6.1 mmol/l (110 mg/dl). Patients with fasting glucose ≥ 7 mol/l (126 mg/dl) at baseline were diagnosed with T2DM. Patients with coronary artery stenosis ≥ 50% were diagnosed with significant coronary artery disease (CAD).

Prospective study:

Patients were followed-up over a period of 8 years (median 7.7 years, range [16 days- 8.8 years]) for cardiovascular-related events including non-fatal myocardial infarction, non-fatal stroke, the need for a coronary artery bypass graft, percutaneous coronary intervention, non-coronary vascular surgery and mortality due to cardiac or vascular reasons.

For the diagnosis of incident T2DM, blood samples were collected at the two, four, six and eight-year visits. Individuals were considered to be diabetic if one of the three criteria was met: fasting glucose levels ≥ 7mmol/l (126 mg/dl), plasma glucose levels ≥ 11.1mmol/l (200 mg/dl) 2 h after an oral glucose challenge (75 g) or the clinical diagnosis of T2DM by a physician during the follow- up period. Subjects were diagnosed to be free from incident T2DM if *both* conditions were met; fasting glucose levels < 7mmol/l (126 mg/dl) *and* plasma glucose levels < 11.1mmol/l (200 mg/dl) 2 h after an oral challenge with 75 g glucose at *all*

the follow-up visits. Subjects with missing values of FPG or 2 h post glucose challenge were excluded. Thus, only 105 subjects from the cohort fulfilled the criteria of incident T2DM (n=35) or the absence of incident T2DM (n=70) and were included in the prospective analysis for T2DM.

Clinical chemistry:

Venous blood samples were collected after overnight fasting and at least 12 h before angiography. Laboratory analysis was performed on fresh serum samples. HbA_{1c} was analysed by high-performance liquid chromatography (HPLC) on a Menarini-Arkray KDK HA 8140 (Arkray KDK, Kyoto, Japan). Triacylglycerols, total cholesterol, HDL and other clinical chemistry variables were measured on a Hitachi 717 or 911 system (Roche).

Sphingolipid analysis:

The sphingoid base profile was analysed as described before [30] with some modifications. Briefly, 0.5 ml methanol including 200 pmol of the internal standards d7-sphingosine and d7-sphinganine (d7SA, d7SO; Avanti Polar Lipids, Alabaster, Alabama, USA) was added to100µl of plasma and extracted for 1h under agitation on a thermo-mixer at 37^{o}C. Precipitated proteins were pelleted by centrifugation and the supernatant transferred to a new tube. For lipid hydrolysis, 75µl of methanolic HCl (1 N HCl and 10 M H_2O in methanol) was added to the supernatant and incubated for 16 hours at 65^{o}C. This was followed by the addition of 100 µl of 10M KOH to neutralize the HCl and hydrolyze the phospholipids. To this mix, 625 µl chloroform was added. Then, 100 µl 2N ammonium hydroxide and 0.5 ml alkaline water were added to complete the phase separation. The mix was then vortexed and centrifuged at 16000 *g* for 5 minutes. After centrifugation, the upper phase was discarded and the lower organic phase was washed two to three times with alkaline water. Finally, the organic phase was dried under N_2 and kept at -20 ^{o}C freezer until analysis.
The sphingoid bases were separated on a C_{18} column (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, Montluçon, France) and analysed on a TSQ Quantum Ultra mass spec (Thermo, Reinach, BL, Switzerland). Each sample was measured as a singleton. Intra- and Inter-assay coefficient of variation (CV %) of the method was between 5% and 20%.

Statistical analysis:

Continuous variables were log-transformed and the means were compared using the two-sided t-test. Categorical variables were compared using the Chi-square test. Prospective analysis of

cardiovascular events was done using Kaplan-Meier curves and Cox hazard regression models (univariate and multivariate). In some cases, Kaplan Meier curves were done after sorting the cohort into two groups above and below the median. The continuous variables were standardized in standard deviation units by subtracting each value from the average and then dividing it by the standard deviation. Hazard ratios are reported per increase of one standard deviation. Univariate Cox regression models were calculated to evaluate the predictive potential of each variable. Multivariate cox regression models were performed to evaluate the predictive role of individual variables after adjusting for other significant variables from the univariate model (with HR lower limit of 95 % CI > 1). Schoenfeld residuals were calculated for each of the covariates and plotted against time-to-event to visually check for the fulfillment of the proportionality assumption and linear regression models using the Schoenfeld residuals as dependent variables were made. None of the covariates showed major violation of the proportionality assumption.

Prospective analysis for the development of T2DM was performed using binary logistic regression. Since only the subjects with confirmed new onset of T2DM (n = 35) or confirmed absence of incident T2DM (n = 70) were selected for the analysis, binary logistic regression was preferred over survival analysis or censored regression methods. Mean comparison was done between the group which developed T2DM and the group which did not develop T2DM. Two-sided t-test was performed on the log-transformed variables. Univariate binary logistic regression models were calculated to evaluate the predictive role of each of the measured variables. Multivariate logistic regression models were then performed to adjust for the traditional predictors. Log transformed and normalized variables in standard deviation units were used for the logistic regression models. The ROC curve analysis was done using the predicted probabilities from some of the univariate and multivariate logistic regression models and the AUCs were compared using Delong`s test. Statistical analyses were performed in SPSS16.0 (IBM, Zurich, Switzerland) and R.

Results:

In a cross-sectional design, we compared sphingoid base plasma profile in individuals with and without MetS (Table 1), in subjects with and without T2DM (Table 2) and in subjects with coronary artery disease (CAD) to CAD-free subjects at baseline (Table 3).

Plasma sphingoid bases are usually *N*-acylated and conjugated to different headgroups, giving rise to the various sphingolipid species. In the current study, we were primarily interested in analysing the sphingoid base profile. Therefore, the total sphingolipids were extracted and acid-hydrolysed to remove the attached *N*-acyl chains and head groups. It should therefore be noted that all reported sphingoid base concentrations are not free sphingoid bases but refer to the sphingoid base content in the total extracted sphingolipids e.g. the reported C_{18}SO concentration reflects all extracted sphingolipids (ceramides, sphingomyelins, glycosphingolipids etc.) which contain a C_{18}SO backbone.

At baseline, plasma 1-deoxySLs and C_{20}-based sphingolipids were significantly higher in patients with MetS (Table 1) (*p = 4.35 * 10 e-7* for 1-deoxySO, *p = 5.08 * 10e-6* for 1-deoxySA, *p = 0.02* for C_{20}SO and *p = 6.047 * 10 e-5* for C_{20}SA), while C_{17}SO, C_{18} SAdiene and C_{18}SO were lower in the plasma of individuals with MetS (*p=0.001, p= 0.009, p= 0.007,* respectively). Sphingoid bases with C_{16} and C_{19} backbones were not different between the groups.

A similar picture is seen for individuals with diagnosed T2DM at baseline (Table2). Also in this group the 1-deoxySLs and C_{20}-based sphingolipids were significantly elevated compared to the non-diabetic subjects (*p* = 9.68 * 10 e-5 for 1-deoxySO; *p* = 0.003 for 1-deoxySA; *p* = 0.034 for C_{20}SO and *p* = 5.05 * 10 e-5 for C_{20}SA) whereas C_{17}SO and C_{18}SAdiene sphingoid bases were lower in patients with T2DM (*p* = 0.043 for C_{17}SO and *p* = 0.013 for C_{18} SAdiene). C_{16}-, C_{19}- and C_{18}- sphingoid bases were not significantly different between these groups

In CAD patients (stenosis of the coronary arteries ≥ 50 %), the levels of C_{17}SO (*p = 0.04)*, C_{18}SAdiene (*p = 8.79 *10 e-4)*, C_{18}SO (*p = 0.001)* and C_{18}SA (*p = 0.002)* were significantly lower at baseline whereas C_{16}, C_{19}, C_{20}- sphingoid bases and 1-deoxySLs were not significantly different among the groups.

Table 1. Baseline characteristics of patients with metabolic syndrome (MetS) and subjects free of MetS. Values are shown as mean ±SD for the continuous variables and numbers and percetnage of total for the categorical variables. *p* values were calculated using the unpaired two sided t-test on the log transformed continuous variables. For the categorical variables, the *p* value was calculated using the Chi square test. Variables in bold font have $p < 0.05$. MetS, metabolic syndrome HOMA-IR, Homeostatic model assessment-insulin resistance, K, serum potassium, CRP, C-reaction protein, GFR (Mayo) Glomerular filtration rate (Mayo equation).

	No MetS at baseline (n= 195)	MetS at baseline (n= 154)	*p*
Age (years)	**64.14 ± 10.2**	**61.18 ± 9.78**	**0.009**
Sex (Female)	66 (33.85%)	46 (29.88%)	0.429
Hypertension (WHO)	**80 (41.03%)**	**101 (65.59%)**	**5.13E-06**
T2DM (baseline)	**32 (16.42%)**	**79 (51.3%)**	**3.68E-12**
History of smoking	**108 (55.39%)**	**103 (66.89%)**	**0.029**
Coronary artery stenoses > 50%	113 (57.95%)	102 (66.24%)	0.114
BMI	**26 ± 4.13**	**28.91 ± 4.14**	**1.13E-10**
Waist circumference (cm)	**91.48 ± 10.71**	**101.37 ± 11.39**	**4.51E-14**
Waist-to-Hip Ratio	**0.93 ± 0.09**	**0.99 ± 0.08**	**3.25E-09**
Cholesterol (mmol/l)	5.64 ± 0.98	5.56 ± 1.25	0.279
LDL (mmol/l)	**3.47 ± 0.81**	**3.26 ± 0.96**	**0.012**
HDL (mmol/l)	**1.41 ± 0.39**	**1.05 ± 0.29**	**5.12E-22**
Triglycerides (mmol/l)	**1.38 ± 0.62**	**2.57 ± 1.59**	**8.99E-24**
Glucose (mmol/l)	**6 ± 1.78**	**7.8 ± 2.63**	**2.49E-16**
HbA_{1c} (%)	**6.04 ± 0.88**	**6.77 ± 1.37**	**1.62E-08**
Insulin (µU/ml)	**9.43 ± 8.27**	**17.26 ± 15.81**	**2.29E-14**
HOMA-IR	**2.55 ± 2.57**	**5.78 ± 4.98**	**9.64E-21**
Systolic BP	**131.19 ± 21.53**	**144.08 ± 20.96**	**9.55E-09**
Diastolic BP	**76.39 ± 12.39**	**82.19 ± 12.05**	**5.97E-06**
CRP	**0.68 ± 0.98**	**1.05 ± 1.64**	**1.18E-04**
K	4.39 ± 0.41	4.41 ± 0.49	0.748
S.Creatinine (mg/dl)	1.13 ± 0.63	1.2 ± 0.74	0.094
GFR (Mayo)	86.26 ± 19.34	85.06 ± 21.99	0.414
T2DM treatment	**32 (16.42%)**	**79 (51.3%)**	**3.68E-12**
Diuretics	**59 (30.26%)**	**64 (41.56%)**	**0.028**
Anti-hypertensive drugs	**151 (77.44%)**	**134 (87.02%)**	**0.022**
Lipid-lowering drugs	93 (47.7%)	82 (53.25%)	0.303
Plasma Sphingolipids			
C_{16}SO (µmol/l)	15.366 ± 5.38	14.275 ± 5.33	0.058
C_{16}SA (µmol/l)	0.447 ± 0.19	0.501 ± 0.289	0.234
C_{17}SO (µmol/l)	**8.162 ± 2.736**	**7.273 ± 2.44**	**0.001**
C_{18}PhytoSO (µmol/l)	0.119 ± 0.042	0.114 ± 0.049	0.103
C_{18}SAdiene (µmol/l)	**28.36 ± 7.81**	**26.31 ± 7.833**	**0.009**
C_{18}SO (µmol/l)	**95.001 ± 18.615**	**89.972 ± 22.446**	**0.007**
C_{18}SA (µmol/l)	3.209 ± 1.047	3.65 ± 1.805	0.075
C_{19}SO (µmol/l)	2.879 ± 1.2	2.696 ± 1.212	0.094
C_{20}SO (µmol/l)	**0.169 ± 0.05**	**0.185 ± 0.064**	**0.020**
C_{20}SA (µmol/l)	**0.025 ± 0.014**	**0.03 ± 0.015**	**6.47E-05**
1-deoxySO (µmol/l)	**0.152 ± 0.086**	**0.211 ± 0.135**	**4.35E-07**
1-deoxySA (µmol/l)	**0.072 ± 0.035**	**0.094 ± 0.057**	**5.08E-06**

Table 2. Baseline characteristics of patients with T2DM compared to T2DM-free subjects of the nested VIVIT cohort. Values are shown as mean ±SD for the continuous variables and numbers and percetnage of total for the categorical variables. *p* values were calculated using the unpaired two sided t-test on the log transformed continuous variables. For the categorical variables, the *p* value was calculated using the Chi square test. Variables in bold font have $p < 0.05$. MetS, metabolic syndrome HOMA-IR, Homeostatic model assessment-insulin resistance, K, serum potassium, CRP, C-reaction protein, GFR (Mayo) Glomerular filtration rate (Mayo equation).

	No T2DM at baseline (n= 238)	T2DM at baseline (n= 111)	*p*	All (n = 349)
Age (years)	63.03 ± 10.29	62.43 ± 9.75	0.682	62.84 ± 10.11
Sex (Female)	79 (33.2%)	33 (29.73%)	0.519	112 (32.1%)
Hypertension (WHO)	124 (52.11%)	57 (51.36%)	0.896	181 (51.87%)
History of smoking	136 (57.15%)	75 (67.57%)	0.064	211 (60.46%)
Coronary artery stenoses > 50%	139 (58.41%)	76 (68.47%)	0.072	215 (61.61%)
MetS ATP III	**75 (31.52%)**	**79 (71.18%)**	**3.68E-12**	154 (44.13%)
BMI	**26.85 ± 4.32**	**28.22 ± 4.36**	**0.005**	27.29 ± 4.37
Waist circumference (cm)	**94.14 ± 11.59**	**99.46 ± 12.23**	**2.22E-04**	95.89 ± 12.05
Waist-to-Hip Ratio	**0.95 ± 0.09**	**0.98 ± 0.09**	**0.002**	0.96 ± 0.09
Cholesterol (mmol/l)	5.67 ± 1.06	5.46 ± 1.18	0.073	5.6 ± 1.11
LDL (mmol/l)	**3.48 ± 0.84**	**3.14 ± 0.95**	**7.60E-04**	3.37 ± 0.89
HDL (mmol/l)	**1.29 ± 0.39**	**1.14 ± 0.36**	**2.26E-04**	1.24 ± 0.39
Triglycerides(mmol/l)	**1.71 ± 1.02**	**2.36 ± 1.69**	**7.01E-05**	1.92 ± 1.3
Glucose (mmol/l)	**5.79 ± 0.92**	**8.94 ± 3.02**	**2.78E-23**	6.8 ± 2.37
HbA_{1c} (%)	**5.82 ± 0.49**	**7.52 ± 1.38**	**5.88E-26**	6.36 ± 1.18
Insulin (µU/ml)	**11.42 ± 12.64**	**16.01 ± 12.59**	**2.56E-05**	12.9 ± 12.78
HOMA-IR	**2.89 ± 2.77**	**6.24 ± 5.43**	**9.17E-16**	3.97 ± 4.14
Systolic BP	135.78 ± 22.23	139.36 ± 22.04	0.138	136.93 ± 22.2
Diastolic BP	78.88 ± 12	79.19 ± 13.72	0.918	78.98 ± 12.56
CRP	**0.79 ± 1.39**	**0.96 ± 1.19**	**0.038**	0.84 ± 1.33
K	**4.36 ± 0.43**	**4.48 ± 0.48**	**0.021**	4.4 ± 0.45
S.Creatinine (mg/dl)	1.15 ± 0.68	1.21 ± 0.68	0.216	1.17 ± 0.68
GFR (Mayo)	86.38 ± 19.02	84.29 ± 23.56	0.268	85.72 ± 20.55
Diuretics	**71 (29.84%)**	**52 (46.85%)**	**0.002**	123 (35.25%)
Anti-hypertensive drugs	**186 (78.16%)**	**99 (89.19%)**	**0.013**	285 (81.67%)
Lipid-lowering drugs	111 (46.64%)	64 (57.66%)	0.055	175 (50.15%)
Plasma Sphingolipids				
C_{16}SO (µmol/l)	15.031 ± 5.553	14.57 ± 4.991	0.419	14.884 ± 5.378
C_{16}SA (µmol/l)	0.463 ± 0.244	0.486 ± 0.233	0.538	0.471 ± 0.24
C_{17}SO (µmol/l)	**7.965 ± 2.737**	**7.351 ± 2.389**	**0.043**	7.77 ± 2.643
C_{18}PhytoSO (µmol/l)	0.118 ± 0.047	0.115 ± 0.042	0.500	0.117 ± 0.045
C_{18}SAdiene (µmol/l)	**28.114 ± 7.934**	**26.043 ± 7.591**	**0.013**	27.455 ± 7.875
C_{18}SO (µmol/l)	93.899 ± 20.869	90.388 ± 19.617	0.105	92.782 ± 20.517
C_{18}SA (µmol/l)	3.274 ± 1.308	3.681 ± 1.678	0.069	3.403 ± 1.446
C_{19}SO (µmol/l)	2.879 ± 1.261	2.624 ± 1.066	0.080	2.798 ± 1.207
C_{20}SO (µmol/l)	**0.172 ± 0.058**	**0.184 ± 0.055**	**0.034**	0.176 ± 0.057
C_{20}SA (µmol/l)	**0.025 ± 0.012**	**0.032 ± 0.02**	**5.05E-05**	0.027 ± 0.015
1-deoxySO (µmol/l)	**0.159 ± 0.098**	**0.218 ± 0.135**	**9.68E-05**	0.178 ± 0.114
1-deoxySA (µmol/l)	**0.076 ± 0.043**	**0.095 ± 0.055**	**0.003**	0.082 ± 0.048

Table 3. Baseline characteristics of patients with coronary artery disease (CAD) and subjects free of CAD. Values are shown as mean ±SD for the continuous variables and numbers and percetnage of total for the categorical variables. *p* values were calculated using the unpaired two sided t-test on the log transformed continuous variables. For the categorical variables, the *p* value was calculated using the Chi square test. Variables in bold font have $p < 0.05$. CAD, coronary artery disease, MetS, metabolic syndrome HOMA-IR, Homeostatic model assessment-insulin resistance, K, serum potassium, CRP, C-reaction protein, GFR (Mayo) Glomerular filtration rate (Mayo equation).

	No significant CAD at baseline (n = 134)	Significant CAD at baseline (n = 215)	*p*
Age (years)	61.806 ± 9.887	63.47 ± 10.21	0.151
Sex (Female)	**64 (47.77%)**	**48 (22.33%)**	**7.4E-07**
Hypertension (WHO)	69 (51.5%)	112 (52.1%)	0.91305
T2DM	35 (26.12%)	76 (35.35%)	0.071771
History of smoking	**64 (47.77%)**	**147 (68.38%)**	**0.000128**
MetS ATP III Definition	52 (38.81%)	102 (47.45%)	0.11406
BMI	**27.989 ± 4.537**	**26.841 ± 4.21**	**0.016**
Waist circumference (cm)	96.191 ± 13.768	95.702 ± 10.865	0.935
Waist-to-Hip Ratio	**0.94 ± 0.101**	**0.961 ± 0.076**	**0.026**
Cholesterol (mmol/l)	5.701 ± 0.969	5.534 ± 1.177	0.080
LDL (mmol/l)	3.418 ± 0.757	3.339 ± 0.955	0.178
HDL (mmol/l)	**1.382 ± 0.452**	**1.148 ± 0.304**	**1.53E-06**
Triglycerides (mmol/l)	**1.763 ± 1.494**	**2.008 ± 1.151**	**0.001**
Glucose (mmol/l)	**6.472 ± 2.009**	**6.992 ± 2.55**	**0.035**
HbA_{1c} (%)	**6.157 ± 1.093**	**6.476 ± 1.212**	**0.007**
Insulin (µU/ml)	11.802 ± 8.877	13.574 ± 14.678	0.439
HOMA-IR	3.348 ± 2.614	4.354 ± 4.818	0.089
Systolic BP	137.724 ± 24.215	136.42 ± 20.855	0.739
Diastolic BP	80.366 ± 12.588	78.095 ± 12.483	0.088
CRP	0.761 ± 1.421	0.891 ± 1.257	0.282
K	4.344 ± 0.419	4.428 ± 0.456	0.089
S.Creatinine (mg/dl)	**1.083 ± 0.547**	**1.212 ± 0.748**	**0.002**
GFR (Mayo)	87.689 ± 19.054	84.462 ± 21.391	0.139
T2DM treatment	35 (26.12%)	76 (35.35%)	0.071771
Diuretics	54 (40.3%)	69 (32.1%)	0.118626
Anti-hypertensive drugs	**101 (75.38%)**	**184 (85.59%)**	**0.016541**
Lipid lowering drugs	**41 (30.6%)**	**134 (62.33%)**	**8.14E-09**
Plasma Sphingolipids			
C_{16}SO (µmol/l)	15.468 ± 4.825	14.521 ± 5.676	0.050
C_{16}SA (µmol/l)	0.489 ± 0.219	0.459 ± 0.252	0.080
C_{17}SO (µmol/l)	**8.088 ± 2.5**	**7.572 ± 2.715**	**0.040**
C_{18}PhytoSO (µmol/l)	0.119 ± 0.039	0.115 ± 0.049	0.176
C_{18}SAdiene (µmol/l)	**29.234 ± 7.801**	**26.347 ± 7.734**	**8.79E-04**
C_{18}SO (µmol/l)	**96.435 ± 17.842**	**90.506 ± 21.751**	**0.001**
C_{18}SA (µmol/l)	**3.643 ± 1.418**	**3.254 ± 1.447**	**0.002**
C_{19}SO (µmol/l)	2.796 ± 1.159	2.799 ± 1.239	0.752
C_{20}SO (µmol/l)	0.176 ± 0.056	0.176 ± 0.058	0.922
C_{20}SA (µmol/l)	0.028 ± 0.017	0.027 ± 0.013	0.476
1-deoxySO (µmol/l)	0.179 ± 0.107	0.177 ± 0.118	0.471
1-deoxySA (µmol/l)	0.084 ± 0.042	0.08 ± 0.051	0.191

A correlation analysis (supplementary table 1) between sphingoid bases, laboratory values and anthropometric measures at baseline revealed a strong positive correlation between 1-deoxySLs (1-deoxySA and 1-deoxySO) and TG, glucose, HbA1c, insulin and HOMA-IR, whereas the other sphingoid bases correlated with LDL and total cholesterol.

Within the analysed cohort, 126 individuals (36%) developed cardiac and vascular events during the follow-up period (Table 4). We therefore evaluated the predictive potential of the sphingoid bases for cardiac and vascular events. In the univariate approach, C_{18}SAdiene and C_{20}SO showed a significant difference from the zero models in Kaplan Meier plots and univariate Cox regression models (Fig. 1 (A, B), supplementary Table 2) while the other sphingoid bases did not show any significant difference.

Table 4: Incident cardiovascular events in the nested VIVIT cohort

	N (%) *total = 349*
Non-fatal myocardial infarction	26 (7.45%)
Non-fatal stroke	19 (5.45%)
Coronary artery bypass graft	14 (4.02%)
Percutaneous coronary intervention	48 (13.76%)
Peripheral vascular surgery(non-coronary)	24 (6.88%)
Mortality due to cardiac or vascular reasons	69 (19.78%)
Total (cumulative)cardiac and vascular events	126 (36.11%)

Multivariate Cox hazard regression models were used to adjust for known cardiovascular risk factors that were significant in the univariate models (i.e. age, sex, smoking, HDL, waist-to-hip ratio, HbA1c, creatinine, GFR, CRP and the extent of coronary artery stenosis as graded by angiography). After adjusting the models to the significant predictors from the univariate analysis, C_{20}SO remained predictive for the risk to develop cumulative cardiovascular events (Fig 1C, HR = 1.31 [95% CI, 1.09 – 1.56], $p = 0.002$) whereas C_{18}SAdiene did not (Fig. 1C). This result is rather surprising since baseline C_{20}SO levels were not significantly different between coronary artery disease patients (as diagnosed by angiography) and controls at baseline (Table 3). This indicates that plasma C_{20}SO levels add a predictive value to the angiographic diagnosis for the development of cardiovascular- related events.

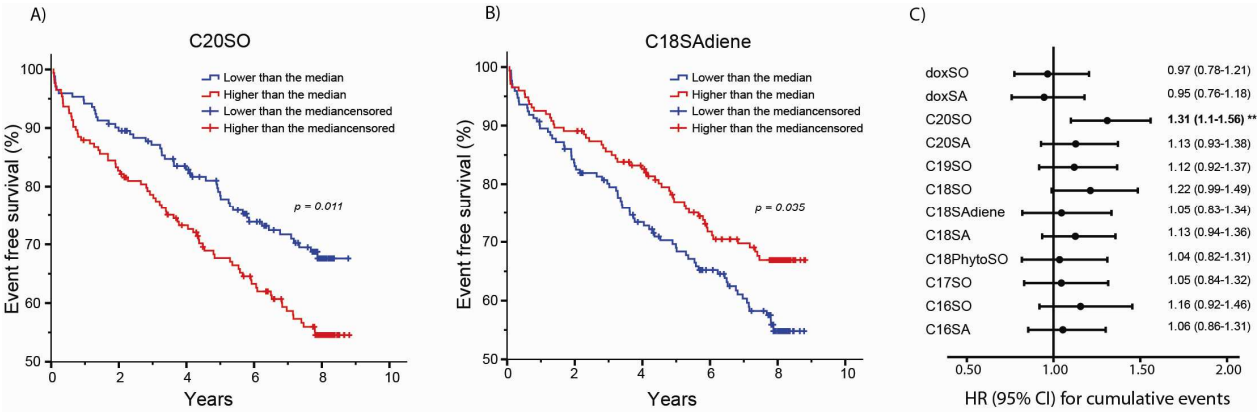

Figure 1. Univariate Kaplan Meier plots (A and B) for plasma C_{20}SO and C_{18}SAdiene. The cohort is sorted into two groups higher (red) and lower (blue) than the median and the *p* values are calculated using log rank test. Forest plot (C) showing the hazard ratio (filled circle) and the 95% CI as horizontal error bar for the individual plasma sphingolipids. Cox hazard regression models were built using the log transformed and standardized variables. Each bar represents a different Cox regression model for each of the sphingolipids and adjusting for age, sex, smoking, HDL, waist-to-hip ratio, HbA_{1c}, creatinine, GFR, CRP and the extent of coronary artery stenosis as graded by angiography

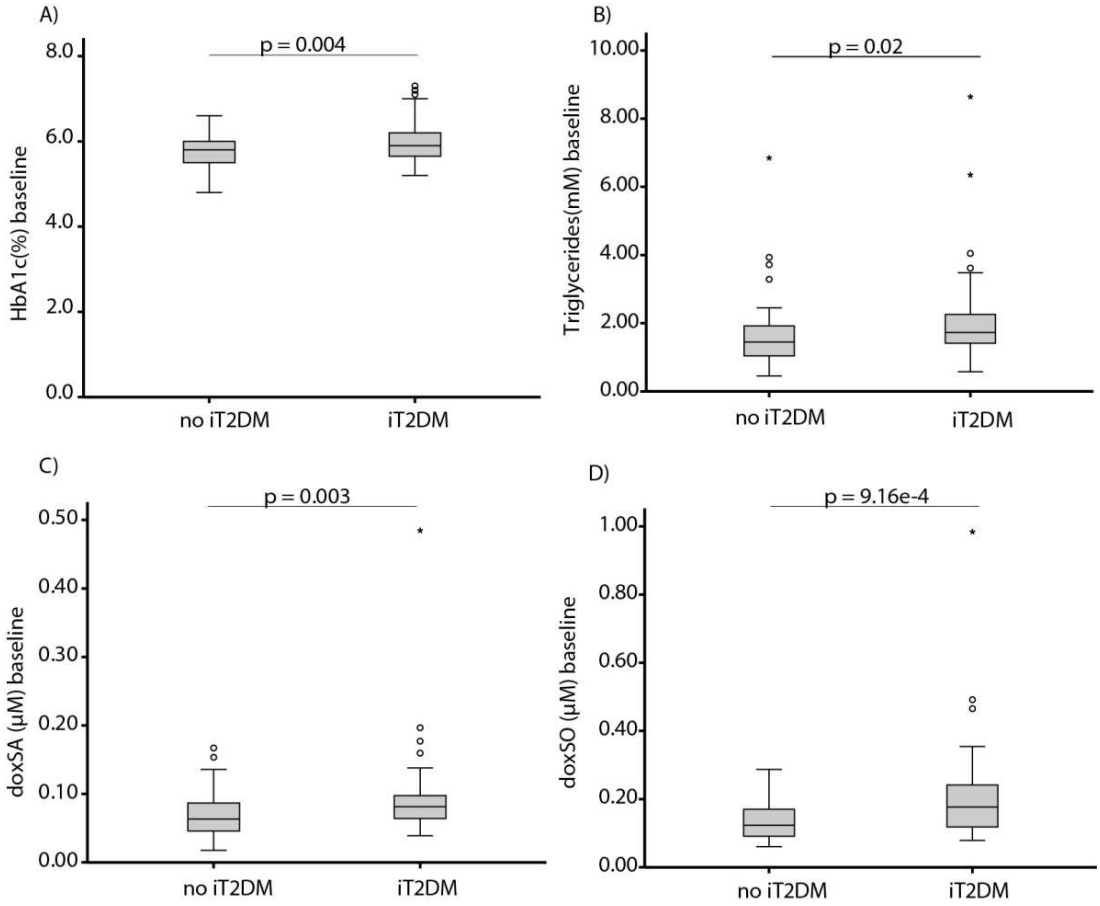

Figure 2. Box and whisker plots showing the plasma values of HbA_{1c} (A), triglycerides (B), 1-deoxySA (C) and 1-deoxySO (D) in the group of patients who developed incident T2DM (iT2DM) and those who did not (no iT2DM). *p* values are calcuated using the t-test on the log transformed values.

The role of the plasma sphingolipids as predictive biomarkers for T2DM development, was assessed by comparing sphingoid base plasma levels at baseline between individuals who developed T2DM during the follow-up period of the study (incident T2DM, n = 35) and those who did not (no-incident T2DM, n = 70). Baseline 1-deoxySLs, HbA_{1c} and triglycerides were significantly elevated in the incident T2DM group (Fig. 2, supplementary table 3), (*p = 9.16 * 10e-4* for 1-deoxySO, *p = 0.003* for 1-deoxySA, *p* = 0.004 for HbA_{1c} and *p* = 0.02 for TGs) in comparison to the no-incident T2DM group. Univariate binary logistic regression (Table 5) revealed that HbA_{1c}, triglycerides, the presence of a metabolic syndrome, potassium, 1-deoxySO and 1-deoxySA were significant predictors for the development of T2DM. To address the predictive role of 1-deoxySLs, we used each of the significant variables in the univariate models as covariates in a bivariate binary logistic regression model in combination with either 1-deoxySO or 1-deoxySA (Table 5).

Table 5. Binary logistic regression models results for incident T2DM showing the odds ratios for the univariate and multivariate models. Variables are log-transformed and standardized in SD units.

		Odds ratio *per one SD increase of the log transformed variable*	*p*
Univariate binary logistic regression models			
	1-deoxySO	2.29 (1.35-3.89)	0.002
	1-deoxySA	1.99 (1.22-3.24)	0.006
	TG	1.69 (1.07-2.68)	0.025
	HbA_{1c}	3.94 (1.47-10.55)	0.007
	MetS	3.47 (1.45-8.31)	0.005
	K	1.77 (1.05-2.97)	0.033
Multivariate binary logistic regression models			
Model 1	MetS	2.74 (1.1-6.83)	0.031
	1-deoxySO	2.09 (1.21-3.63)	0.009
Model 2	1-deoxySO	1.92 (1.05-3.53)	0.037
	TG	1.25 (0.73-2.15)	0.426
Model 3	1-deoxySO	2.25 (1.29-3.91)	0.004
	HbA_{1c}	3.75 (1.34-10.48)	0.012
Model 4	1-deoxySO	2.04 (1.15-3.65)	0.016
	K	1.58 (0.92-2.7)	0.098
Model 5	HbA_{1c}	3.64 (1.28-10.37)	0.016
	1-deoxySO	2.05 (1.15-3.64)	0.015
	MetS	2.63 (1.02-6.77)	0.047

Baseline 1-deoxySO, HbA1c and the presences of a metabolic syndrome remained significant after this analysis, while 1-deoxySA turned non-significant after adjusting to triglycerides (supplementary table 4). Even after adjusting for HbA1c and MetS status, 1-deoxySO remained statistically significant (Table 5) with an odds ratio of 2.05 ([CI 95%, 1.15-3.64], $p = 0.015$).

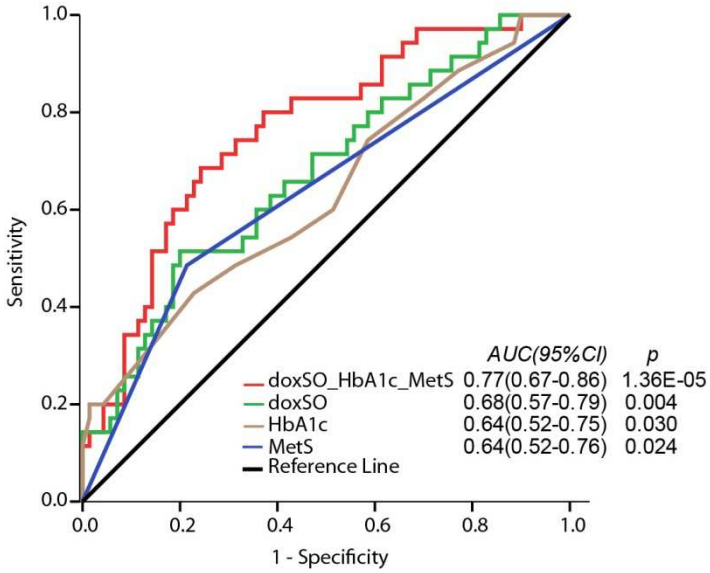

Figure 3. Receiver operating characteristics (ROC) curves showing the area under the curve for 1-deoxySO, HbA_{1c} and MetS and the combined model based on the logistic regression model to discriminate the incident T2DM group from those who did not develop T2DM. Areas under the curve (AUCs) and their 95% CIs are shown. *p* values are calculated for the difference from the reference line (an AUC of 0.5).

A ROC curve analysis, using the predicted probabilities from the univariate logistic regression models for 1-deoxySO, HbA_{1c} and metabolic syndrome showed similar areas under the curves for the individual variables (AUC = 0.68, 0.64 and 0.64 respectively). However, the ROC curve for the multivariate model including all three variables showed a significant improvement from the univariate ROC curves (AUC = 0.77, $p = 0.011$ compared to HbA_{1c} only and $p = 0.013$ compared to MetS only).

Discussion:

We found in two previous cross sectional studies that 1-deoxySLs are elevated in the plasma of patients with metabolic syndrome and T2DM [33, 34]. These previous findings were also confirmed in the current study and extended by the observation that plasma 1-deoxySLs are predictors for the risk to develop T2DM. The ROC curve analysis showed that 1-deoxySLs, in particular 1-deoxySO, has a comparable diagnostic value to some of the traditional risk factors such as HbA_{1c} or the presence of a metabolic syndrome. Importantly, we showed that the combination of 1-deoxySO with the presence of metabolic syndrome and HbA_{1c}

significantly improved the ability to discriminate the individuals who developed T2DM during the follow-up from those who did not. Pathologically elevated plasma 1-deoxySLs were originally reported in the context of the rare inherited sensory neuropathy HSAN1 (hereditary sensory neuropathy type I) which is associated with several missense mutations in the SPT genes *SPTLC1* and *SPTLC2* [35-37]. These mutations induce a shift in the substrate specificity of the enzyme that leads to increased 1-deoxySL formation [37]. Further studies showed that 1-deoxySLs are neurotoxic and were found to accumulate preferentially in the peripheral nerves but not in the brain or other organs of transgenic HSAN1 animals [37, 38]. Moreover, it was shown that the formation of 1-deoxySLs can be efficiently suppressed in-vitro and in-vivo by an oral supplementation with L-serine [38]. HSAN1 mice which received an L-serine-enriched diet did not develop neuropathic symptoms whereas a supplementation with alanine aggravated the neuropathic symptoms in these animals [38]. In a pilot study with HSAN1 patients, we demonstrated that an oral L-serine supplementation was also effective in lowering deoxy-SL levels in humans [38]. However, it is not fully understood why 1-deoxySLs are also elevated in conditions of metabolic disorders such as MetS or T2DM, despite the absence of mutations in SPT. Interestingly, Elevated plasma 1-deoxySL levels were also found in plasma of pre-diabetic and diabetic monkeys where the animals were fed high fat and high fructose diet to induce obesity and T2DM [39]. This corroborates our findings that 1-deoxySLs are elevated early in the course of T2DM development. Since 1-deoxySLs are cytotoxic in culture it cannot be excluded that they are also mechanistically involved in the development of insulin resistance, β-cell failure or other hallmarks of T2DM pathogenesis. The finding that 1-deoxySLs might be involved in the pathogenesis of HSAN1 suggests that these lipids might also be involved in the pathogenesis of the diabetic sensory neuropathy (DSN) especially since both conditions have a very similar clinical picture.

In addition, we investigated the associations of the plasma sphingoid base profile with atherosclerotic cardiovascular disease. We found that the plasma levels of C_{18}SA, C_{18}SO, and C_{18}SAdiene were significantly lower in patients with coronary artery disease whereas plasma 1-deoxySLs were not altered in these conditions. Interestingly, C_{20}SO-based sphingolipids, albeit not significantly different between patients with and without CAD at baseline, emerged in multivariate analysis as independent predictors for the risk to develop cardiovascular events. This was still true after adjusting for the degree of coronary artery stenosis. The decrease in plasma C_{18}SA C_{18}SO, C_{18}SAdiene in CAD patients appears , on the first glance, to be in contrast to earlier findings which reported increased plasma sphingomyelin (SM) levels in patients with coronary artery disease [16, 17]. However, other studies with the aim to

evaluate plasma SM as a predictive risk marker for cardiovascular events, either in patients with stable angina or in the general adult population, did not show any significant predictive role [18, 19]. It is noteworthy that, our study focuses on the analysis of the total sphingoid base profile by LC-MS after a complete chemical hydrolysis of the extracted plasma sphingolipids. In contrast, sphingomyelin levels were analyzed by an enzymatic method [40] which is based on the release of phosphocholine after enzymatic hydrolysis. Since sphingomyelins are heterogeneous and not only composed of C_{18} sphingoid bases, the reported sphingomyelin levels represent the total sum of all sphingomyelins whereas our analysis reflects the sum of all sphingolipids with a specific sphingoid base composition, independently of the *N*-acyl chains and head groups. Although ~95% of the sphingolipids in plasma are sphingomyelins [41], only 60% of the total plasma sphingomyelins contain C_{18} sphingosine backbone [41]. It is therefore conceivable that the distinct sphingoid base composition of sphingomyelin subspecies is not detected by the enzymatic assay.

To our surprise, we found that C20-sphingosine was predictive for the risk to develop cardiovascular events although the cross sectional comparison at baseline showed no difference between CAD patients and controls. C20-sphingoid bases are minor constituents of plasma of plasma sphingolipids and reflect less than 1% of the total sphingoid bases in plasma. The presence of a C20SO backbone was first described in the brain gangliosides of rats and humans [42], in gangliosides of human stomach mucosa [43] and in sphingomyelin of rat liver [44]. They were shown to increase with aging in human and rat brains [42, 45]. Using imaging mass spectrometry, C20-based sphingolipids were shown to exist in distinct areas of the rat hippocampus depending on age [46]. It was also demonstrated that the feeding of radiolabeled palmitoyl-CoA did not yield any radiolabeled C20-based sphingolipids while feeding cells with radiolabeled stearoly-CoA yielded both C18-based and C20-based sphingolipids [47]. This indicates that the labeled stearoly-CoA is used directly as a substrate for SPT to form C20-based sphingolipids and it can also be metabolized to palmitoyl-CoA which acts as a precursor for C18-based sphingolipids. It is not fully understood how the substrate shift in SPT is regulated but it has been suggested that certain factors might play a role. In particular the SPT small subunit B (ssSPTb) has been shown to increase the condensation of stearoly-CoA when over-expressed with SPTLC1 and either the SPTLC2 or SPTLC3 subunits [11]. Moreover, It has also been shown that the presence of SPTLC3 enables the SPT enzyme to metabolize distinct acyl-CoAs Interestingly, in two recent GWAS studies variants of the *SPTLC3* gene were associated with an increased risk for myocardial infarction [49] and with differences in the plasma levels of several sphingomyelin species

[50]. This further supports our finding that C20-based sphingolipids are independent predictors for cardiovascular events

With respect to the limitation of the current study, it is important to consider that the analyzed cohort might not faithfully represent the situation in the general population. The extrapolations of the risk predictions might be done only to clinical settings where the prevalence of cardiometabolic disease is high. Moreover, the number of events, especially for the development of T2DM, is not sufficient to adjust for all possible confounders without over-fitting. Thus, we cannot fully exclude the effect of other confounders.

In summary, we have demonstrated that sphingolipids with different backbones show promising predictive roles in cardiovascular disease or T2DM independent of the traditional risk factors. However, ultimately larger and interventional studies are needed to confirm the potential of these markers in risk re-classification and the effect on patient management.

Supplementary information:

Table S1. Heat map showing the Spearman correlation coefficients for the analyzed plasma sphingolipids bases and the clinical chemistry and anthropometric variables in the nested VIVIT cohort. Bold font signifies $p < 0.05$. The color code is shown with the lowest value of -1 (blue) and the highest value of 1(red)

	C_{16}SO	C_{16}SA	C_{17}SO	C_{18}PhytoSO	C_{18}SAdiene	C_{18}SO	C_{18}SA	C_{19}SO	C_{20}SO	C_{20}SA	1-deoxySO	1-deoxySA
Age	**0.12**	-0.03	**0.23**	0.09	**0.11**	0.10	-0.06	**0.25**	-0.05	-0.05	**-0.14**	**-0.18**
BMI	0.06	**0.16**	-0.08	-0.03	0.02	-0.04	**0.20**	-0.07	0.09	**0.18**	**0.30**	**0.26**
waist circum.	-0.11	0.04	**-0.25**	**-0.15**	**-0.12**	**-0.14**	0.10	**-0.20**	0.03	0.10	**0.33**	**0.26**
WHR	**-0.25**	-0.08	**-0.38**	**-0.20**	**-0.27**	**-0.20**	-0.02	**-0.27**	0.00	0.02	**0.27**	**0.16**
Cholesterol	**0.42**	**0.36**	**0.40**	**0.42**	**0.54**	**0.57**	**0.38**	**0.19**	**0.22**	**0.20**	**0.19**	**0.25**
LDL-C	**0.34**	**0.31**	**0.37**	**0.38**	**0.45**	**0.51**	**0.29**	**0.20**	**0.18**	**0.14**	0.05	**0.12**
HDL-C	**0.38**	0.06	**0.39**	**0.30**	**0.44**	**0.33**	0.07	**0.16**	-0.10	-0.09	-0.09	-0.03
TG	-0.08	**0.14**	-0.16	-0.02	-0.06	-0.07	**0.14**	**-0.12**	**0.15**	**0.22**	**0.43**	**0.40**
Glucose	-0.04	0.08	**-0.15**	-0.06	**-0.17**	-0.10	**0.15**	-0.08	**0.16**	**0.26**	**0.34**	**0.25**
HbA_{1c}	0.02	-0.02	-0.01	0.07	-0.03	0.02	0.09	-0.04	0.04	**0.12**	**0.21**	**0.22**
Insulin	0.04	**0.18**	-0.06	-0.04	-0.07	-0.05	**0.18**	0.02	**0.22**	**0.20**	**0.23**	**0.20**
HOMA	0.03	**0.20**	-0.09	-0.01	-0.10	-0.07	**0.19**	-0.02	**0.23**	**0.26**	**0.30**	**0.26**
Systolic BP	0.05	0.07	0.04	0.07	0.04	0.01	0.07	0.00	0.05	**0.13**	0.02	0.07
Diastolic BP	0.02	0.09	-0.03	0.02	-0.01	-0.05	0.06	-0.02	0.05	0.09	0.10	**0.13**
CRP	**-0.12**	-0.04	-0.05	-0.02	-0.01	**0.13**	**0.15**	0.01	**0.14**	0.03	0.05	**0.11**
K	-0.04	-0.06	-0.08	-0.01	0.02	0.03	0.01	-0.09	0.00	-0.03	**0.16**	**0.15**
S. Creatinine	-0.11	**-0.16**	-0.10	-0.08	**-0.16**	-0.10	**-0.19**	-0.02	-0.04	**-0.13**	0.07	0.04
GFR (Mayo)	**-0.11**	0.09	**-0.22**	-0.05	**-0.11**	-0.06	**0.12**	**-0.20**	0.07	**0.12**	0.07	0.07

Spearman rho						
-1	-0.5	-0.25	0	0.25	**0.5**	**1**

Table S2. Univariate Cox hazard regression models for the development of total cardiovascular events. Continuous variables are log-transformed and standardized into units of standard deviation (SD)**.** Thus hazard ratios are expressed for an increase of one unit SD.

	HR (95% CI) *per 1 SD*	p
Age	1.18(0.99-1.42)	0.080
Sex (male)	**2.09(1.36-3.19)**	**7.42E-04**
Hypertension	1.07(0.76-1.52)	0.715
T2DM	1.29(0.9-1.86)	0.174
History of smoking	**1.58(1.09-2.29)**	**0.017**
Coronary artery stenoses > 50%	**2.78(1.83-4.23)**	**1.94E-06**
Extent of stenosis	**1.26(1.15-1.38)**	**6.99E-07**
MetS ATP III Definition	1.29(0.91-1.83)	0.160
DM drugs	1.29(0.9-1.86)	0.174
Diuretics	1.16(0.81-1.66)	0.441
HTN drugs	1.36(0.84-2.21)	0.225
Lipid lowering drugs	1.33(0.93-1.88)	0.121
BMI	0.9(0.75-1.08)	0.247
Hip circumference	**0.8(0.66-0.98)**	**0.027**
Waist circumference (cm)	1.08(0.9-1.29)	0.432
Waist-to-Hip ratio	**1.31(1.09-1.57)**	**0.005**
Cholesterol	**0.8(0.67-0.96)**	**0.012**
LDL	**0.83(0.69-1)**	**0.042**
HDL	**0.75(0.62-0.91)**	**0.003**
Triglycerides	1.02(0.86-1.21)	0.868
Glucose	1.17(1-1.37)	0.052
HbA_{1c}	**1.26(1.08-1.47)**	**0.004**
Insulin	1.11(0.96-1.28)	0.193
HOMA-IR	1.1(0.93-1.3)	0.295
Systolic BP	1.04(0.87-1.24)	0.694
Diastolic BP	0.99(0.83-1.18)	0.897
CRP	**1.15(1.01-1.31)**	**0.047**
K	1.19(1-1.43)	0.060
S.Creatinine	**1.32(1.2-1.46)**	**9.23E-08**
GFR (Mayo)	**0.75(0.63-0.89)**	**8.63E-04**
C_{16}SO	0.9(0.75-1.08)	0.247
C_{16}SA	0.87(0.72-1.07)	0.168
C_{17}SO	0.85(0.71-1.02)	0.072
C_{18}PhytoSO	0.87(0.72-1.06)	0.143
C_{18}SAdiene	**0.79(0.66-0.95)**	**0.011**
C_{18}SO	0.96(0.81-1.15)	0.642
C_{18}SA	0.95(0.79-1.15)	0.593
C_{19}SO	1.02(0.86-1.21)	0.857
C_{20}SO	**1.18(1.02-1.37)**	**0.028**
C_{20}SA	0.98(0.82-1.18)	0.810
1-deoxySO	1(0.84-1.18)	0.932
1-deoxySA	0.95(0.79-1.14)	0.553

Table S3. Baseline values of clinical, lab and sphingolipid levels in the incident T2DM group and the group which did not develop T2DM until the end of the study period (8 years). *p* values are calculated using the t-test on the log transformed variables. Variables in bold have a $p < 0.05$.

	no incident T2DM (n= 70)	incident T2DM (n= 35)	p
Age (years)	61.286 ± 9.133	64.486 ± 10.309	0.137
Sex (Female)	26 (37.15%)	13 (37.15%)	1.000
Hypertension (WHO)	28 (40%)	21 (60%)	0.053
History of smoking (baseline)	37 (52.86%)	16 (45.72%)	0.490
Coronary artery stenoses > 50%	38 (54.29%)	23 (65.72%)	0.263
MetS ATP III Definition	**15 (21.43%)**	**17 (48.58%)**	**0.004**
BMI	26.419 ± 3.325	27.142 ± 5.129	0.642
Waist circumference (cm)	92.492 ± 10.499	96.157 ± 13.184	0.187
Waist-to-Hip Ratio	0.928 ± 0.087	0.953 ± 0.103	0.243
Cholesterol (mmol/l)	5.649 ± 1.05	5.795 ± 1.299	0.677
LDL (mmol/l)	3.476 ± 0.802	3.437 ± 0.891	0.695
HDL (mmol/l)	1.305 ± 0.395	1.277 ± 0.4	0.682
Triglycerides(mmol/l)	**1.61 ± 0.93**	**2.2 ± 1.62**	**0.020**
Glucose (mmol/l)	5.752 ± 1.062	6.023 ± 1.198	0.241
HbA_{1c} (%)	**5.723 ± 0.391**	**6.006 ± 0.547**	**0.004**
Insulin (µU/ml)	8.98 ± 4.605	15.826 ± 26.165	0.324
HOMA-IR	2.281 ± 1.246	2.895 ± 2.203	0.537
Systolic BP	131.595 ± 19.088	139.559 ± 20.501	0.053
Diastolic BP	76.545 ± 10.971	79.853 ± 13.623	0.261
CRP	0.698 ± 0.96	0.785 ± 1.486	0.792
K	**4.289 ± 0.395**	**4.462 ± 0.322**	**0.029**
S.Creatinine (mg/dl)	1.053 ± 0.149	1.231 ± 0.999	0.361
GFR (Mayo)	89.486 ± 15.349	85.536 ± 22.558	0.257
Diuretics	20 (28.58%)	14 (40%)	0.238
Anti-hypertensive drugs	**57 (81.43%)**	**34 (97.15%)**	**0.026**
Lipid lowering drugs	37 (52.86%)	16 (45.72%)	0.490
C_{16}SO (µmol/l)	15.272 ± 5.339	16.48 ± 8.635	0.875
C_{16}SA (µmol/l)	0.46 ± 0.186	0.531 ± 0.409	0.858
C_{17}SO (µmol/l)	7.96 ± 2.781	8.75 ± 3.753	0.382
C_{18}PhytoSO (µmol/l)	0.112 ± 0.036	0.134 ± 0.085	0.352
C_{18}SAdiene (µmol/l)	28.375 ± 7.547	30.828 ± 11.845	0.552
C_{18}SO (µmol/l)	93.077 ± 19.409	101.702 ± 31.292	0.163
C_{18}SA (µmol/l)	3.28 ± 1.121	3.682 ± 2.081	0.387
C_{19}SO (µmol/l)	2.796 ± 1.136	3.228 ± 1.544	0.220
C_{20}SO (µmol/l)	0.168 ± 0.052	0.192 ± 0.081	0.128
C_{20}SA (µmol/l)	0.024 ± 0.009	0.028 ± 0.017	0.365
1-deoxySO (µmol/l)	**0.14 ± 0.062**	**0.213 ± 0.167**	**9.16E-04**
1-deoxySA (µmol/l)	**0.069 ± 0.032**	**0.098 ± 0.077**	**0.003**

Table S4. Binary logistic regression model results for incident T2DM showing the odds ratios for the multivariate models including plasma 1-deoxySA as a predictor.

Multivariate binary logistic regression models		OR *per 1 SD of log transformed variable*	p
Model 1	MetS	2.63 (1.05-6.57)	0.039
	1-deoxySA	1.77 (1.06-2.94)	0.029
Model 2	1-deoxySA	1.68 (0.97-2.88)	0.064
	TG	1.34 (0.8-2.27)	0.279
Model 3	1-deoxySA	1.87 (1.14-3.09)	0.015
	HbA_{1c}	3.47 (1.26-9.53)	0.016
Model 4	1-deoxySA	1.74 (1.03-2.95)	0.040
	K	1.67 (0.98-2.84)	0.062
Model 5	1-deoxySA	1.66 (0.99-2.8)	0.058
	HbA_{1c}	3.44 (1.23-9.64)	0.019
	MetS	2.59 (1.01-6.69)	0.050

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Chapter 3

Oral L-Serine Supplementation Lowers Plasma 1-Deoxysphingolipids in Experimental Diabetic Rats and Improves Diabetic Neuropathy*

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Contribution statement

AO was involved in the study designed the experiment, did the lipid extraction, mass spectrometric analysis, the statistical analysis and wrote the manuscript. IA and YW did the lipid extraction and tissue homogenization. RB, CPS were involved in the study design, performed the animal experiment, phenotyping, neurobehavioral and neurophysiological tests. GLP was involved in the study design. AvE contributed to study design, data interpretation and critically revised the manuscript. TH was involved in study design, data interpretation and supervision and critically revised the manuscript.

*** Manuscript in preparation**

Abstract:

Background:

1-Deoxysphingolipds (1-deoxySLs) are neurotoxic sphingolipids which are formed by the enzyme serine-palmitoyltransferase (SPT) due to the promiscuous use of L-alanine instead of its canonical substrate L-serine. Pathologically elevated 1-deoxySLs are associated with the rare hereditary sensory and autonomic neuropathy type 1 (HSANI) , which is caused by several missense mutations in the SPT. Plasma 1-deoxySLs are also elevated in patients with metabolic syndrome (MetS) or type 2 diabetes mellitus (T2DM). HSAN1 and the diabetic sensory neuropathy are clinically very similar and are characterized by a painful, progressive and length-dependent axonopathy which typically starts in the distal extremities. We have previously shown that the pathological 1-deoxySLs formation in HSAN1 is significantly suppressed in response to an oral L-serine supplementation. In this context, we investigated whether a serine supplementation is also beneficial for the treatment of the diabetic neuropathy in an experimental diabetic rat model.

Methods:

A streptozotocin (STZ)-induced diabetic rat model was used to investigate the effects of serine on the diabetic neuropathy. STZ was injected intra-peritoneally to induce pancreatic β cell failure and type 1 diabetes mellitus. Vehicle-injected rats were used as controls. The effect of serine was tested in preventive and therapeutic schemes. In the preventive approach, control (n= 8) and STZ rats (n= 10) had immediate access to either a standard or a serine-enriched diet for 18 weeks after the STZ injection. In the therapeutic schedule, animals were fed a standard diet for the first 8 weeks after STZ injection and were then randomized into either a control group receiving a standard diet (n= 8) or a treatment group receiving a serine enriched diet (n= 10). Plasma and tissue sphingolipids were quantified using LC/MS after lipid extraction and acid hydrolysis. Thermal, mechanical nociception and nerve conduction velocity (NCV) were measured.

Results:

In both treatment schemes (therapeutic and preventive), plasma 1-deoxySLs were significantly lowered in the serine-supplemented diabetic animals ($p < 0.0001$). Serine had no significant effect on hyperglycemia, body weight and food intake. Neurologically, the serine supplemented animals showed significantly improved mechanical sensitivity in both, the preventive ($p < 0.01$) and therapeutic groups ($p < 0.001$). NCV was significantly better in the

preventive but not in the therapeutic group (30.3 m/sec ± 2.2 (serine diet) vs 23.8 m/sec ± 1.0 (standard diet), $p < 0.05$). NCV showed a highly significant inverse correlation with plasma 1-deoxySL levels (p = 5.2E-12).

Conclusion:

Oral serine supplementation effectively lowered the plasma 1-deoxySLs in diabetic STZ rats and significantly improved the mechanical sensitivity and nerve conduction velocity in these animals. This suggests that oral serine supplementation could be a potential therapeutic strategy in diabetic neuropathy.

Abbreviations:

1-deoxySA	1-deoxysphinganine
1-deoxySO	1-deoxysphingosine
1-deoxySLs	1-deoxysphingolipids
$C_{16\text{-}19}$	Carbon chain length (16 carbons -19 carbons)
DRG	Dorsal root ganglia
HSAN1	Hereditary sensory and autonomic neuropathy type I
MetS	Metabolic syndrome
NCV	Nerve conduction velocity
SA	Sphinganine
SO	Sphingosine
SPT	Serine palmitoyltransferase
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus

Introduction:

Diabetic neuropathy is the most common complication of type 1 and type 2 diabetes mellitus affecting up to 50 % of these patients [1, 2]. It affects single or multiple nerves (mononeuropathy or polyneuropathy) with sensory, motor or autonomic involvement, leading to a wide variety of clinical presentations. Distal symmetrical polyneuropathy (DSN) is the most common presentation of diabetic neuropathy [3]. It usually starts in the lower extremities with either negative (e.g. numbness) or positive symptoms (e.g. neuropathic pain) or both at the same time. Symptoms are mainly sensory and symmetrical on both sides with a "stocking and gloves" distribution. This is frequently associated with bad wound healing and ulcers (diabetic foot), which may require amputations and results in a significant reduction in the quality of life [4].

DSN is caused by an axonopathy of both, myelinated and non-myelinated fibers due to micro-angiopathy or direct nerve injury. Injuries to the endothelium of the micro-vessels lead to a decreased O_2 supply to the nerve [5] which is a documented feature of the diabetic neuropathy. However, it is not clear whether the ischemia itself or an associated pathology causes the neuropathy since it was shown that the blood flow in sciatic nerves of diabetic rats was not altered compared to healthy animals [6]. On the contrary, the number of micro-vessels in the peripheral nerves of diabetic rats was shown to be increased [7] in the early stages of diabetic neuropathy.

In addition to the micro-angiopathy, direct nerve injury and injury of the nerve supporting cells (e.g. Schwan cells in the PNS or satellite cells in the DRGs) are established pathologies associated with DSN. In that context, several pathogenic mechanisms for the direct nerve injury have been proposed. Hyperglycemia is considered as an essential contributor in the pathogenesis of diabetic polyneuropathy (DPN). Excess glucose can be converted to sorbitol leading to osmotic and oxidative stress [8]. Glucose can also be shunted to the hexosamine pathway leading to modification of transcription factors and an inflammatory response [9]. Moreover, the increased flux of glucose through the glycolytic chain and TCA cycle leads to oxidative stress by overloading the respiratory capacity of mitochondria [10]**.** Long term and persistent hyperglycemia leads also to non-enzymatic modifications of proteins and the formation of the pro-inflammatory advanced glycation end products (AGEs) [11]. All these factors are believed to participate in the pathogenesis of DSN to a variable extent.

Although hyperglycemia plays a vital role in the pathogenesis of DSN, the tight control of plasma glucose levels is not sufficient to prevent or reverse diabetic peripheral neuropathy. Intensive glycemic control in T1DM patients delayed the onset and the progression of diabetic neuropathy only in 60% of the patients [12]. These findings were replicated in different cohorts with different follow-up periods [3]. Thus, a significant proportion of T1DM patients still developed neurological symptoms despite intensive glycemic control. Moreover, in T2DM, intensive glycemic control failed to show a robust effect in delaying the progression of diabetic neuropathy [13, 14]. This suggests that other factors, apart from the hyperglycemia, are underlying the pathogenesis of the diabetic neuropathy. Dyslipidemia, in particular hypertriglyceridemia, might be one of these additional factors. In the European Diabetes Prospective Complications Study (EURODIAB), hypertriglyceridemia was identified as an independent predictor for the development of diabetic neuropathy in T1DM even after adjusting for the duration of diabetes and HbA1c [15]. Interestingly, in T1DM dyslipidemia develops usually after a period of sustained or uncontrolled hyperglycemia [16] which coincides with the course of the neuropathy in T1DM patients [17]. In T2DM patients, dyslipidemia appears early and might even precede the onset of hyperglycemia. Furthermore, hypertriglyceridemia has been shown to correlate with the progression of diabetic neuropathy independent of the diabetes control which further supports the view that dyslipidemia plays an independent role in diabetic neuropathy [18]**.** Pre-diabetes and metabolic syndrome, where dyslipidemia is more prevalent, might also contribute to the risk and pathogenesis of the diabetic neuropathy[19].

Besides triglyceride, other lipid classes might also be involved in the pathogenesis of diabetes and its sequelae. Sphingolipids have been identified over the last years as emerging players in insulin resistance and T2DM [20]. Sphingolipids constitute a heterogeneous class of lipids and are essential components of the plasma membrane and plasma lipoproteins. Sphingolipid de-novo synthesis starts in the ER with the condensation of palmitoyl-CoA and serine – a reaction catalyzed by the enzyme serine palmitoyltransferase (SPT). The product 3-ketosphinganine is rapidly reduced to sphinganine (C_{18}SA). C_{18}SA can be further metabolized to ceramides which are the building blocks for complex sphingolipids such as sphingomyelin and glycosphingolipids. Upon degradation, complex sphingolipids are hydrolyzed to ceramides which, in turn, are degraded to sphingosine (C_{18}SO). Apart from these two canonical substrates, SPT can also utilize other acyl-CoAs in the range of C_{12}-C_{18} and other amino acids such as alanine or glycine thereby generating a diverse class of atypical sphingoid bases. SPT is a multimeric enzyme which is composed of three subunits SPTLC1,

SPTLC2 and SPTLC3 [21, 22]. Several missense mutations in the *SPTLC1* and *SPTLC2* genes [23] are associated with the rare and inherited sensory neuropathy HSAN1 (hereditary sensory and autonomic neuropathy type I) [24-26]. HSAN1 presents as a painful, length-dependent axonal neuropathy and shows very similar symptoms to the diabetic sensory neuropathy. Typical symptoms include loss of sensation, parasthesia, tactile allodynia and painless ulcers and starts usually in feet and hands and progresses proximally over time. The HSAN1-causing mutations in the SPT lead to increased activity with alanine and glycine which results in the formation of an atypical class of 1-deoxySLs that lack the C_1 hydroxyl group of the serine-based sphingoid bases. Consequently, 1-deoxySLs are not metabolized to complex sphingolipids nor degraded by the canonical pathway which requires the formation of sphingosine-1-phosphate as a catabolic intermediate. 1-deoxySL levels are significantly elevated in the plasma of HSAN1 patients and plasma and nerves of a transgenic HSAN1 mouse model [25, 27]. 1-deoxySLs were shown to be neurotoxic and to reduce the number and length of neurites *in vitro* [25, 27]. Interestingly, elevated 1-deoxySLs were also found in patients with the metabolic syndrome and T2DM, despite the absence of mutations in the SPT [28, 29]. Elevated 1-deoxySL levels were also observed in the plasma and liver of STZ rats [29]. In analogy to HSAN1, these elevated 1-deoxySL levels in T2DM might also be involved in the pathogenesis of the diabetic sensory neuropathy. We previously showed that an oral supplementation with L-serine results in a significant reduction of the 1-deoxySLs in HSAN1 mouse models and HSAN1 patients [27]. Serine-supplemented HSAN1 mice were protected and did not develop any neuropathic manifestations [27]. On the basis of these findings, we were interested to test whether a serine supplementation is also effective in lowering plasma 1-deoxySL in the diabetic context in an experimental animal model for diabetic neuropathy and whether such a treatment also results in improved neuropathy in these animals.

Methods:

Animal experiments:

Male Sprague-Dawley rats (180-200 g, Charles River, Calco, Italy) were used for the current study. The animals had access to food and water ad libitum. Diabetes was induced in overnight-fasted rats by a single intra-peritoneal injection of 60 mg/kg streptozotocin (STZ) (Sigma, St. Louis, MO) dissolved in sodium citrate buffer (pH 4.5). Control rats were injected with sodium citrate buffer (pH 4.5) only. Hyperglycemia was confirmed by measuring glycosuria 48 h after STZ injection (Keto-Diabur test, Roche Diagnostics, Spa, Italy). Blood glucose was determined after tail bleeding using the Ascensia Elite assay (Bayer, Basel,

Switzerland). Food and water intake were assessed at the specified time points by averaging over 2-days period. At the end of the study, animals were sacrificed, the tissues were dissected and immediately frozen in liquid N_2.

Experimental design:

In this study, two experimental designs were employed (Fig1.). In the preventive schedule, the animals had immediate access to either a serine-enriched diet (containing 10% L- serine for the control group and 5% serine for the STZ group) or a standard diet (Mucedola s.r.l, Milan, Italy) immediately after the STZ injection. In the therapeutic schedule, animals remained on a standard diet for 8 weeks after STZ injection and were then randomized into groups receiving either a serine-enriched (10% for the control group or 5% for the STZ group) or a standard diet. The serine content in the food for the STZ animals was half since it is known that the STZ rats consume double the amount of food compared to the control animals.

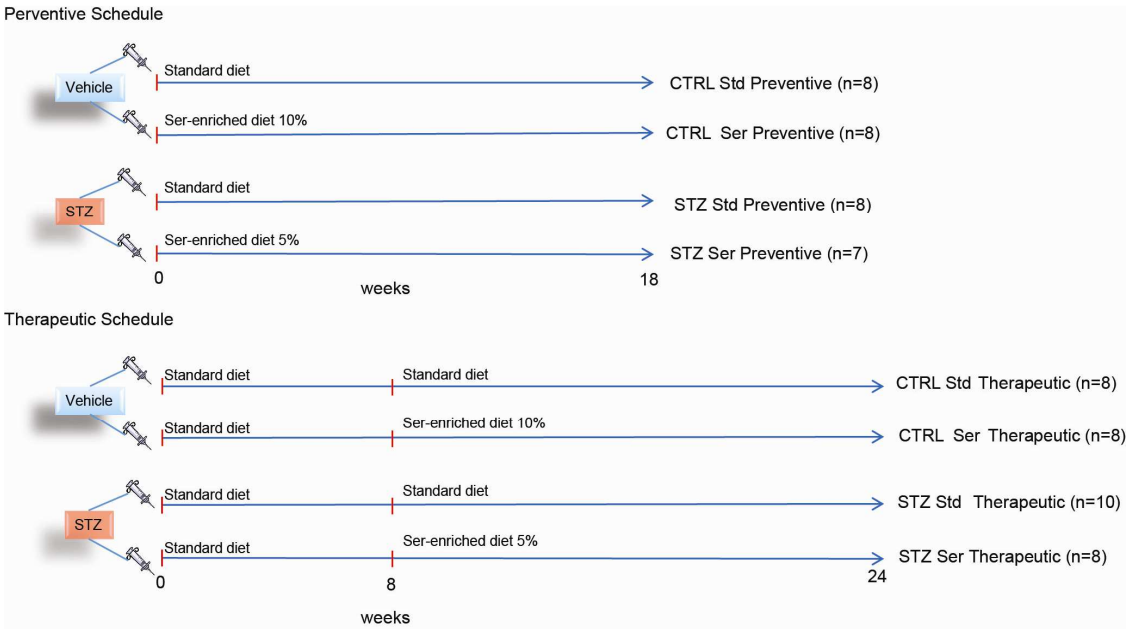

Figure 1. Schematic diagam showing the flow chart of the exmperiment. Animals were injected either STZ 60mg/kg i.p or vehicle at day 0. In the preventive schedule, at day 0 , a serine-enriched or a standard diet was introduced then the animals were followed for 18 weeks post-STZ injection untill they were sacrificied. In the therapuetic scheudle, animals were fed a standrad diet for 8 weeks then they were randomized into either a serine-enriched diet or a standard diet groups where they were followed until the end of the study period at week 24 post-STZ.

Behavioural tests and electrophysiology:

Thermal and mechanical nociception were assessed as behavioral measures for the diabetic neuropathy. The nociceptive threshold to radiant heat was quantified using the hot-plate paw

withdrawal test [30]. In brief, a 40-cm-high Plexiglas cylinder was suspended over the hot plate, and the temperature was maintained at 50°C to give a latency period of approximately 10 s for control rats. Withdrawal latency was defined as the time between placing the rat on the hot plate and the time of withdrawal and licking the hind paw (or manifesting discomfort). Mechanical allodynia on the plantar surface of the rat was assessed by a dynamic paw withdrawal test with a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy), which generates a linearly increasing mechanical force. The paw withdrawal reflex was recorded automatically by measuring the latency until withdrawal in response to the applied force.

Nerve Conduction Velocity, NCV, was measured as described previously [30]. In brief, the anti-dromic sensory NCV in the tail nerve was assessed by placing recording ring electrodes distally in the tail. Stimulating ring electrodes were placed 5 and 10 cm proximally from the recording point. The latencies of the potentials, recorded at the two sites after nerve stimulation, were determined (peak to peak), and NCV was calculated. All of the neurophysiological determinations were performed under standard conditions and at a controlled temperature (room and animals). Core temperature was maintained at 37°C by using heating pads and lamps.

Sphingolipid analysis:

The lipids were analysed as described before [29] with some modifications. Briefly, 100µl plasma or 80 ug proteins from the tissue homogenate was added to 0.5 ml of methanol and spiked with 200 pmol of the internal standards d7-sphingosine and d7-sphinganine (d7SA, d7SO; Avanti Polar Lipids, Alabaster, Alabama, USA). Tissues were homogenized in Precellys® homogenization tubes in (PBS pH =7.4 with 0.2% Triton X-100 (vol/vol)) using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The mix was then incubated in thermo-mixer at 37^{o}C for one hour. Precipitated proteins were pelleted by centrifugation and the supernatant transferred to a new tube. For lipid hydrolysis, 75µl of methanolic HCl (1 N HCl and 10 M H_2O in methanol) was added to the supernatant and incubated for 16 hours at 65^{o}C. This was followed by the addition of 100 µl of 10M KOH to neutralize the HCl and hydrolyze the phospholipids. To this mix, 625µl chloroform was added. Then, 100 µl 2N ammonium hydroxide and 0.5 ml alkaline water were added to complete the phase separation. The mix was then vortexed and centrifuged at 16000*g* for 5 minutes. After centrifugation, the upper phase was discarded and the lower organic phase was washed two to three times with alkaline water. Finally, the organic phase was dried under N_2 and kept at -20 ^{o}C freezer until analysis.

The sphingoid bases were separated on a C_{18} column (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, Montluçon, France) and analysed on a TSQ Quantum Ultra mass spec (Thermo, Reinach, BL, Switzerland). Each sample was measured as a singleton. Intra- and Inter-assay coefficient of variation (CV %) of the method was between 5% and 20%.
In this study 9 different sphingoid base backbones were analysed. The plasma and tissue levels of C_{16}SO, C_{17}SO, C_{18}SO, C_{18}SA, C_{18}SAdiene, C_{18}PhytoSO, C_{20}SO, C_{20}SA, and 1-deoxySA sphingoid bases were quantified.

Statistical analysis:
Data are shown as mean ± SEM. For normally distributed variables, one way ANOVA is performed followed by the Bonferroni correction for the multiple comparisons. In the Bonferroni correction, only four comparisons were considered, control on standard diet vs. control on serine diet; control on standard diet vs. streptozotocin on standard diet, control on serine diet vs. streptozotocin on serine diet and streptozotocin on standard diet vs. streptozotocin on serine diet. Variables which were not normally distributed were log-transformed. The statistical analysis was done in SPSS 16.0 (IBM, Zurich, Switzerland) and GraphPad Prism 5.04 (GraphPad Software, Inc., San Diego, CA).

Results:

Serine-enriched diet does not affect the body weight, hyperglycemia or food intake in STZ rats.

STZ-treated animals developed hyperglycemia (Fig. 2C-D, 3B) within 48 hours post-injection. Hyperglycemia (400 mg/dl - 800 mg/dl or 22.2 mmol/l - 44.4 mmol/l) persisted in the STZ group until the end of the study (preventive 18 weeks; therapeutic 24 weeks). Serine supplementation did not affect the hyperglycemia. In contrast to controls and despite of an increased food and water intake, the STZ group failed to gain body weight (Fig. 2A-B, 3A). Serine supplementation had no influence on body weight, food or water consumption in the STZ and control groups (Fig. 2 E-H, 3C-D).

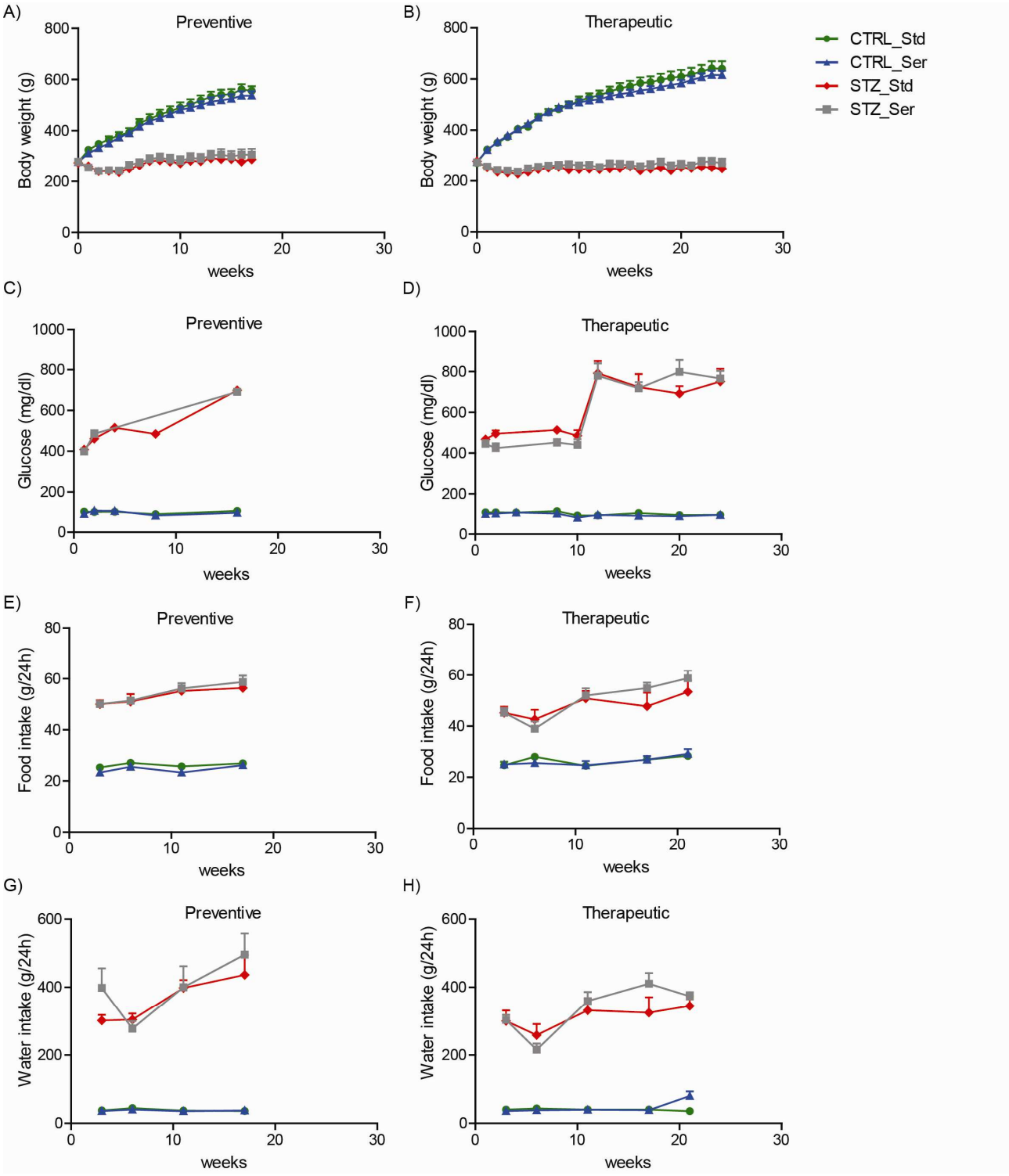

Figure 2. Time course of general phenotype characteristics. Line plots showing the body weight (A-B), blood glucose (C-D), food (E-F) and water intake (G-H) of the animal used in the study. The plots show the values over the entire period of the preventive (Left) and therapeutic (right) schedules for the different groups. The values are expressed as mean ± SEM. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet.

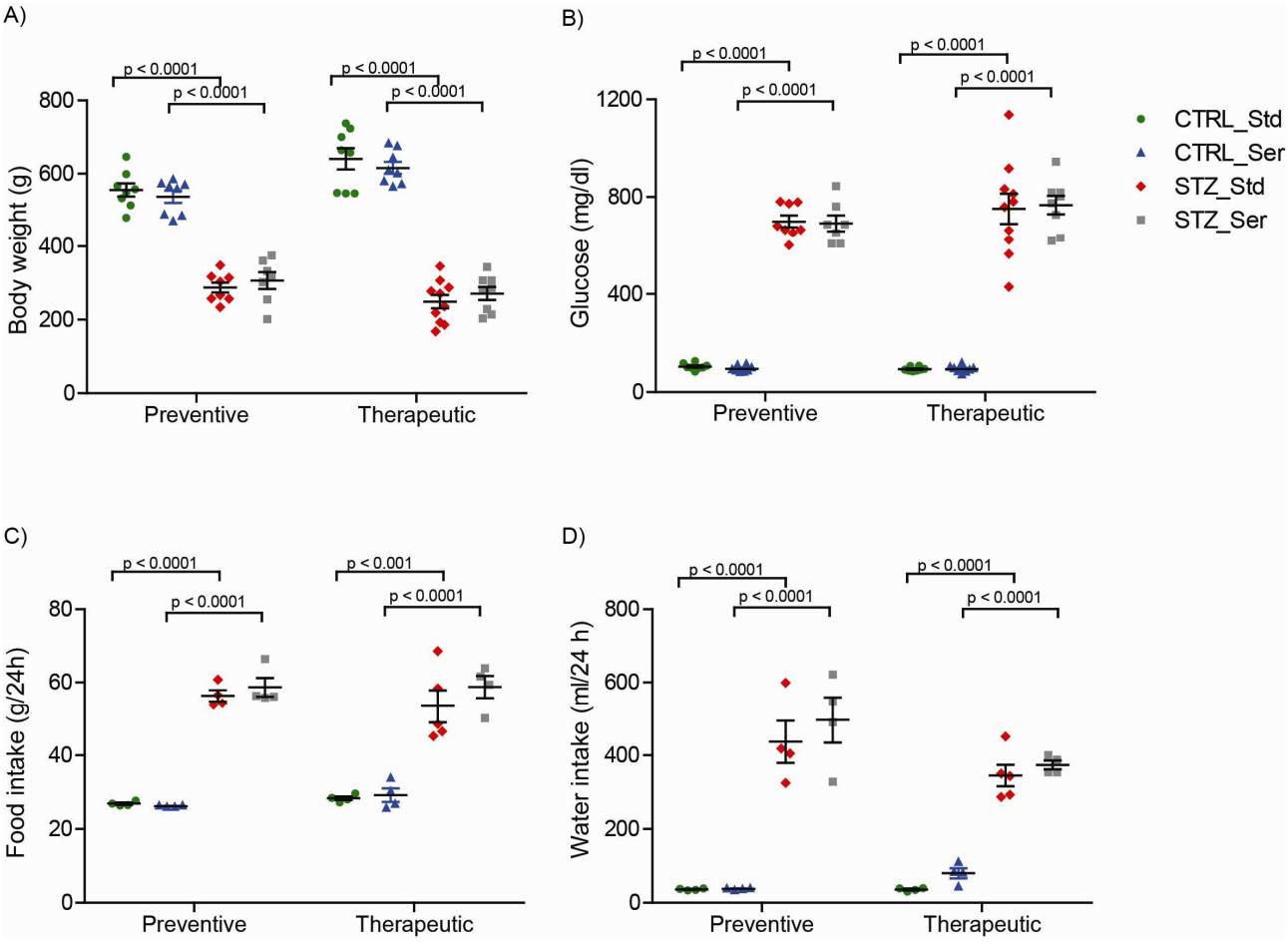

Fig 3. Phenotype characteristics at the last time point before sacrifice.. Scatter plots show the values of the body weight (A), blood glucose (B), food (C) and water intake (D) at week 16 post-STZ injection for the preventive group and week 24 post-STZ injection for the therapeutic group. The values are expressed as mean ± SEM. *p* values are calculated using ANOVA followed by the Bonferroni correction comparing CTRL_Std to CTRL_Ser, CTRL_Std to STZ_Std, CTRL_Ser to STZ _Ser and STZ_Std to STZ_Ser. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet.

Serine enriched diet lowers 1-deoxysphingolipids in the plasma of STZ rats without affecting the other typical and atypical sphingolipids

Sphingoid bases in plasma are usually conjugated to a variety of fatty acids and different head groups. In the current analysis, we were primarily interested in the sphingoid base profile as a measure of the heterogeneous SPT activities. Therefore, the *N*-acyl chains and the head groups of the extracted plasma sphingolipids were removed by acid hydrolysis and the free sphingoid bases were analysed by LC-MS. The herein reported sphingoid base concentrations are therefore a measure for the total concentrations of sphingolipids which are formed on this specific sphingoid base backbone.

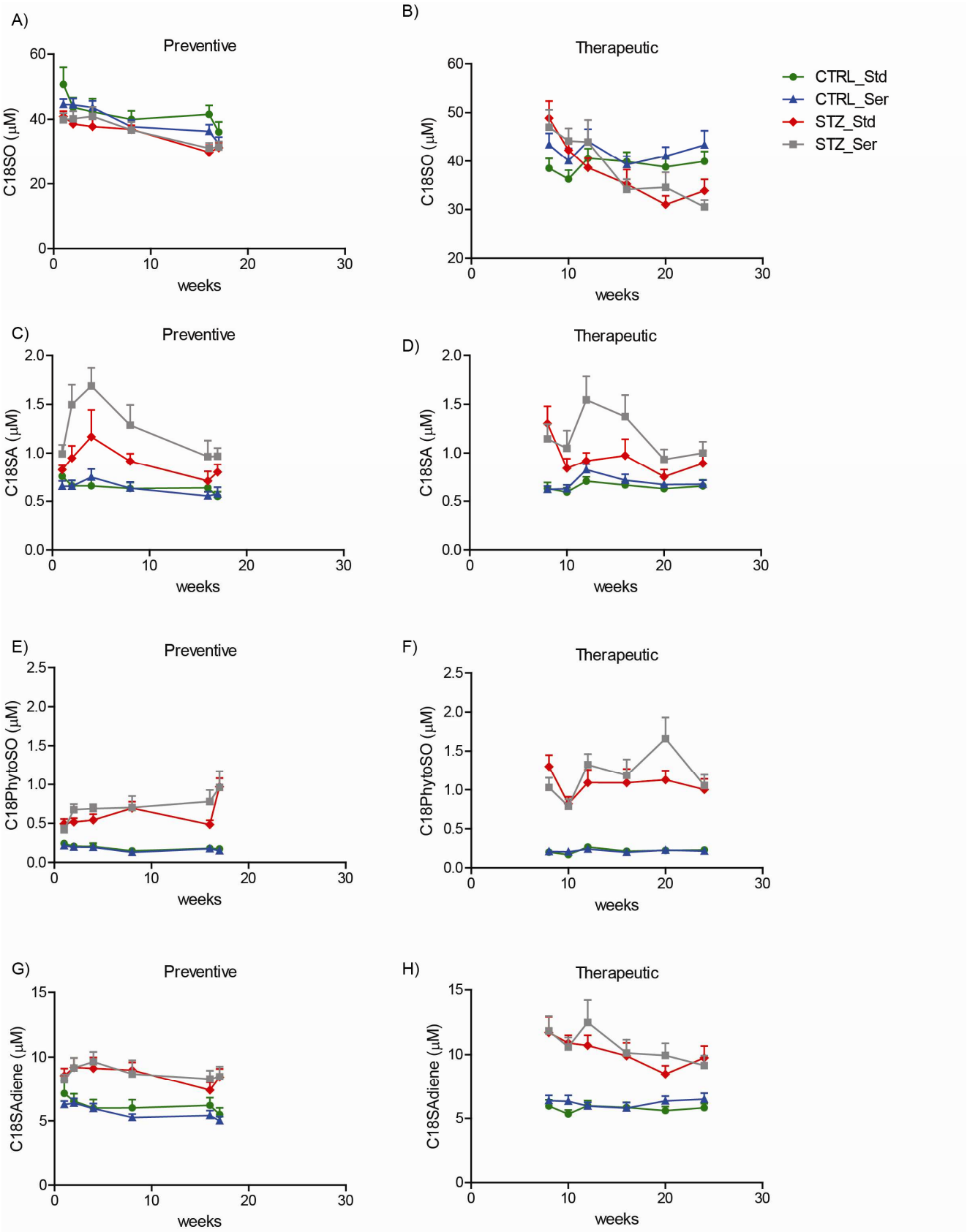

Figure 4. Time course of plasma levels of typical sphingolipids. Line plots show the plasma levels of C_{18}SO (A-B), C_{18}SA (C-D), C_{18}PhytoSO-based sphingolipids (E-F) and C_{18}SAdiene (G-H) over the entire period of the preventive (Left) and therapeutic (right) schedules for the different groups. The values are expressed as mean ± SEM. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet

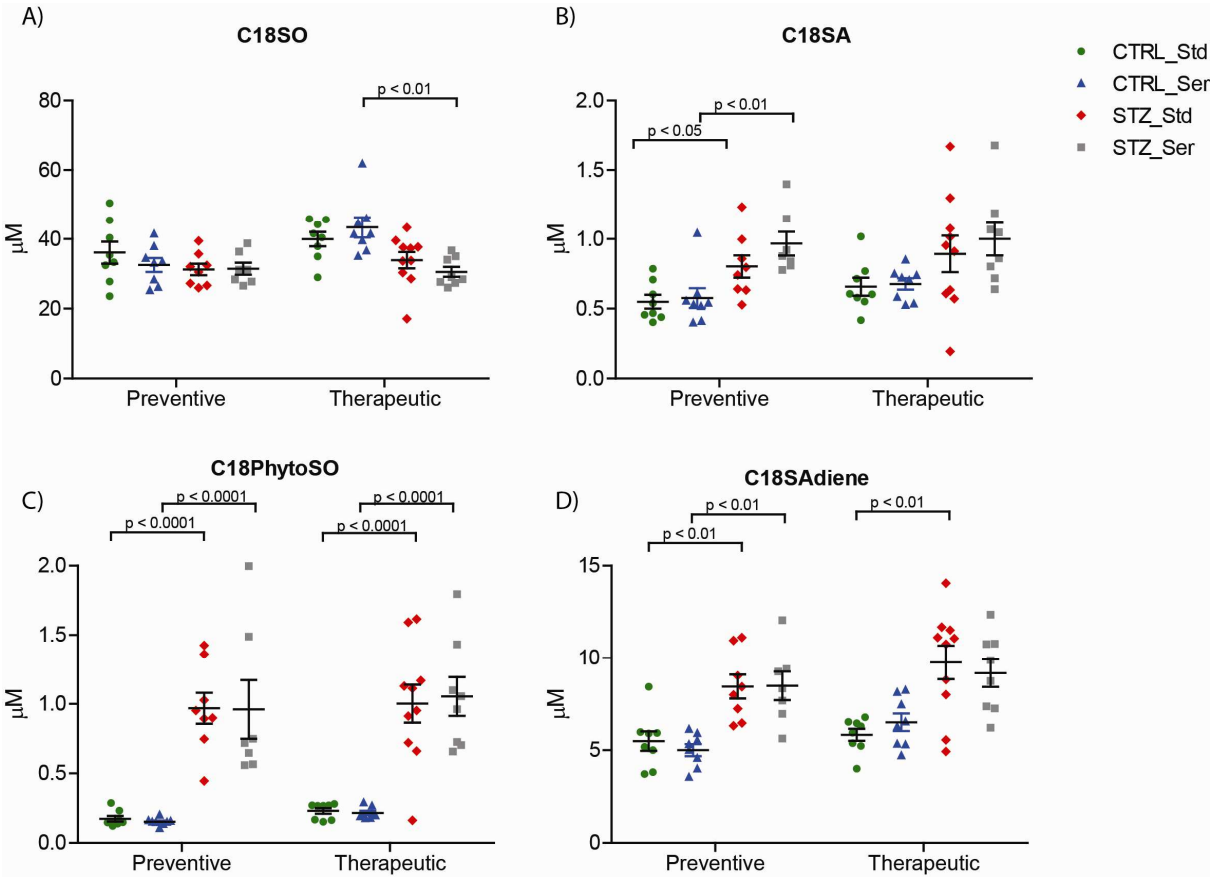

Figure 5. Plasma levels of typical sphingolipids at the last time point before sacrifice. Scatter plots show the values for C_{18}SO (A), C_{18}SA (B), C_{18}PhytoSO (C) and C_{18}SAdiene (D) at week 17 post-STZ for the preventive group and week 24 post-STZ for the therapeutic groups. The values are expressed as mean ± SEM. *p* values are calculated using ANOVA followed by the Bonferroni correction comparing CTRL_Std to CTRL_Ser, CTRL_Std to STZ_Std, CTRL_Ser to STZ _Ser and STZ_Std to STZ_Ser. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet

Sphingolipids containing a C_{18}SO backbone were the dominant forms in plasma but were not significantly different among the groups (Fig. 4A-B, 5A). C_{18}SA increased initially in the serine-supplemented STZ animals, reaching a maximum after 3-4 weeks and then decreased again, although the levels remained slightly elevated until the end of the study (Fig.4 C-D, 5B). C_{18}-SAdiene and C_{18}PhytoSO (Fig. 4E-H, 5C-D) were significantly elevated in all diabetic groups but did not change upon serine supplementation.

Plasma 1-deoxySA was significantly elevated in the STZ rats on standard diet but remained low in the serine supplemented STZ rats. In the preventive schedule, the plasma 1-deoxySA was significantly higher in the STZ animals on standard diet ($p < 0.0001$) compared to the other groups (Fig. 6A-B, 7E). 1-deoxySA increased from 0.03 µmol/l ± 0.003 at the beginning of the study to reach 0.05 µmol/l ± 0.007 before sacrifice in the STZ rats on standard diet (Fig 6A, 7E) whereas the levels remained low in the serine-fed STZ rats until the end of the preventive scheme (0.016 µmol/l ± 0.001 at week 17 post-STZ injection).

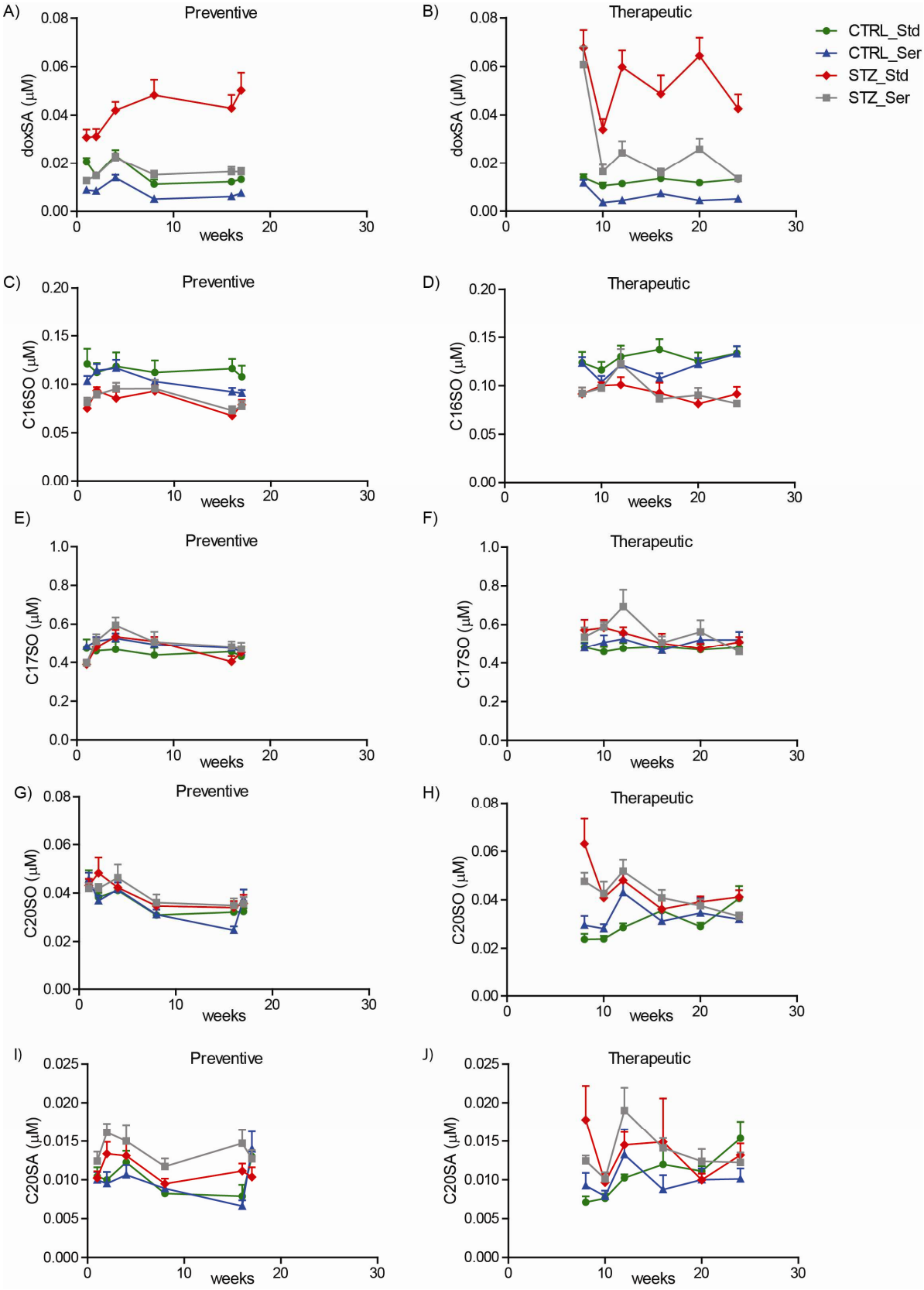

Figure 6. Time course plasma levels of atypical sphingolipids. Line plots show plasma levels of 1-deoxySA (A-B).C_{16}SO (C-D), C_{17}SO (E-F), C_{20}SO (G-H), C_{20}SA-based sphingolipids (I-J) over the entire period of the preventive (Left) and therapeutic (right) schedules for the different groups. The values are expressed as mean ± SEM. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet

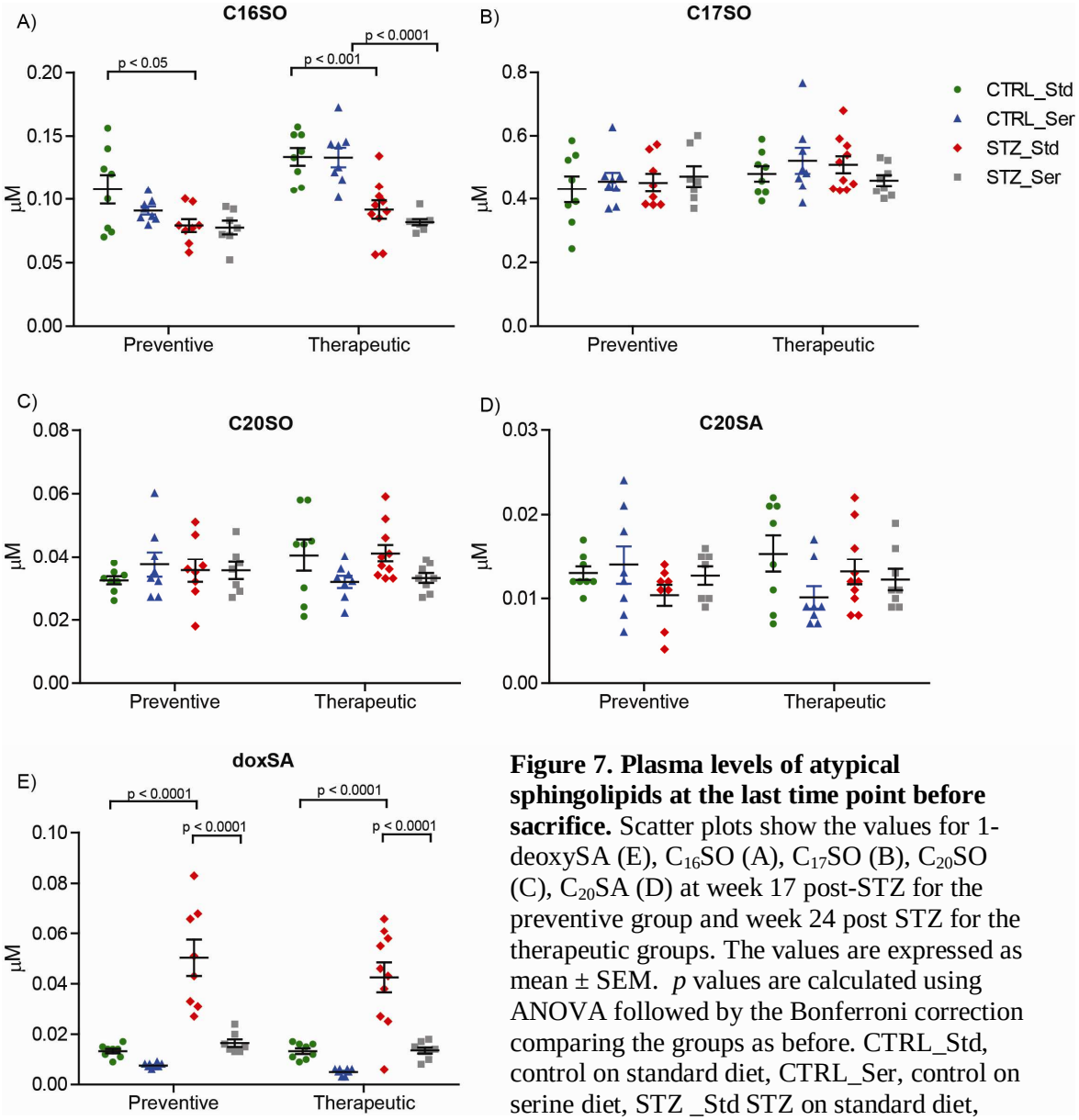

Figure 7. Plasma levels of atypical sphingolipids at the last time point before sacrifice. Scatter plots show the values for 1-deoxySA (E), C_{16}SO (A), C_{17}SO (B), C_{20}SO (C), C_{20}SA (D) at week 17 post-STZ for the preventive group and week 24 post STZ for the therapeutic groups. The values are expressed as mean ± SEM. *p* values are calculated using ANOVA followed by the Bonferroni correction comparing the groups as before. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet

For the therapeutic scheme, we observed a rapid decrease of 1-deoxySA to the control levels after switching to the serine-enriched diet in week 8 whereas 1-deoxSA levels remained high in the diabetic rats on standard diet (Fig. 6B). This effect remained significant until the end of the therapeutic protocol (Fig. 6E). No significant difference was observed between the control rats with and without serine supplementation.

C_{16}SO (Fig. 6 C-D, 7A) was not significantly different among the groups at the beginning but was significantly lower in the STZ groups compared to vehicle controls in both schemes at the end of the study. C_{17}SO, C_{20}SO and C_{20}SA did not change among all groups (Fig .6C-J, 7B-D).

In contrast to the plasma, 1-deoxySLs were not detected in the nervous tissues including sciatic nerve, spinal cord, dorsal root ganglia, neither of control nor of diabetic animals (Fig. 8). Comparing the sphingoid bases distribution within these tissues revealed that the sciatic nerve and dorsal root ganglia had a similar sphingoid base distribution. In contrast, the proportion of C_{18}SA and C_{17}SO was higher while the proportion of C_{18}SAdiene was lower in the neuronal tissue compared to the plasma

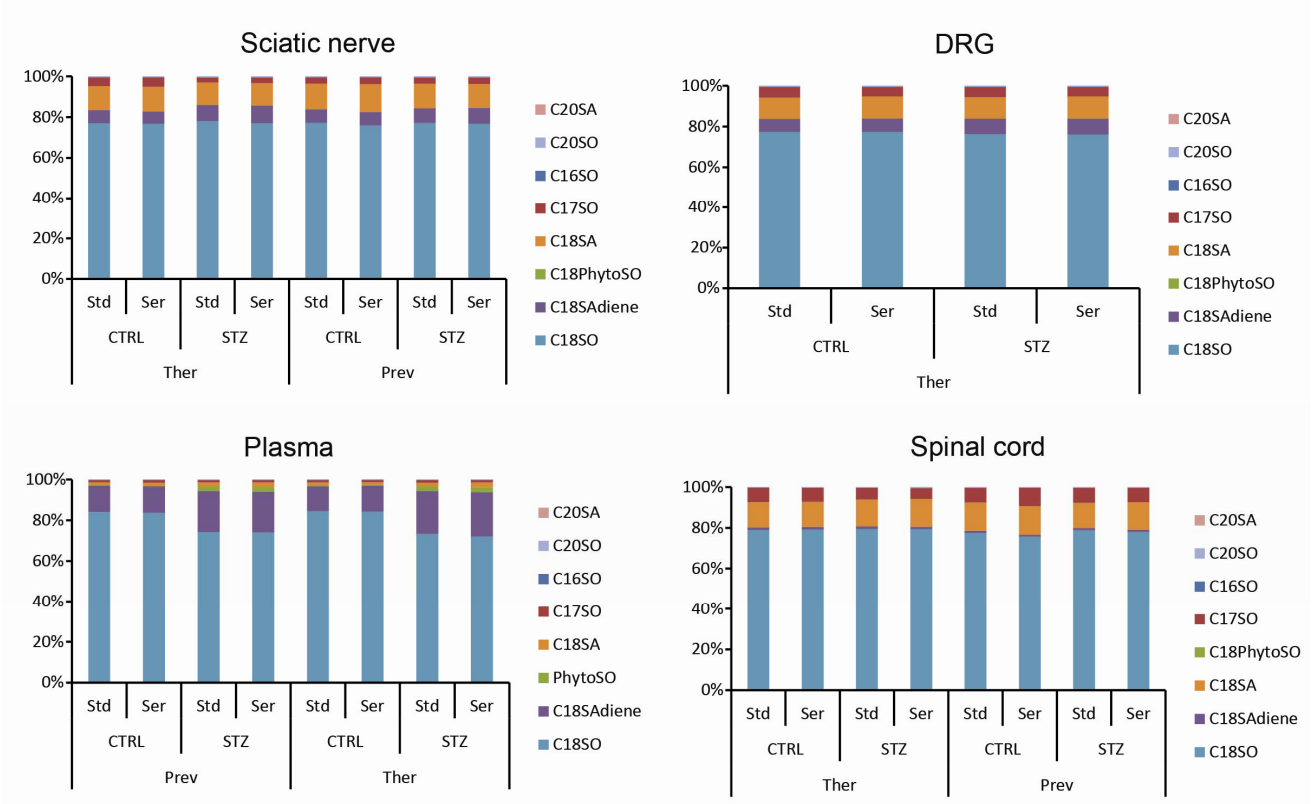

Figure 8. Sphingolipid distribution by backbone in the plasma, sciatic nerve, dorsal root ganglia (DRGs) and spinal cord. The total sphingolipid content is set to 100% and the relative ratio of each sphingoid base backbone is shown. . CTRL, control animals, STZ, streptozotocin animals, Std standard diet, Ser, serine diet, Prev, preventive scheme, Ther, therapeutic scheme.

Serine-enriched diet improves the diabetic neuropathy phenotype in STZ rats

Although the STZ groups showed by trend an increased thermal response latency (Fig. 9A-B), the average thermal sensitivity at the end of the study was not significantly different between the STZ and vehicle treated animals on standard diet (Fig .10A) Therefore, the effect of serine supplementation on thermal sensitivity could not be assessed.

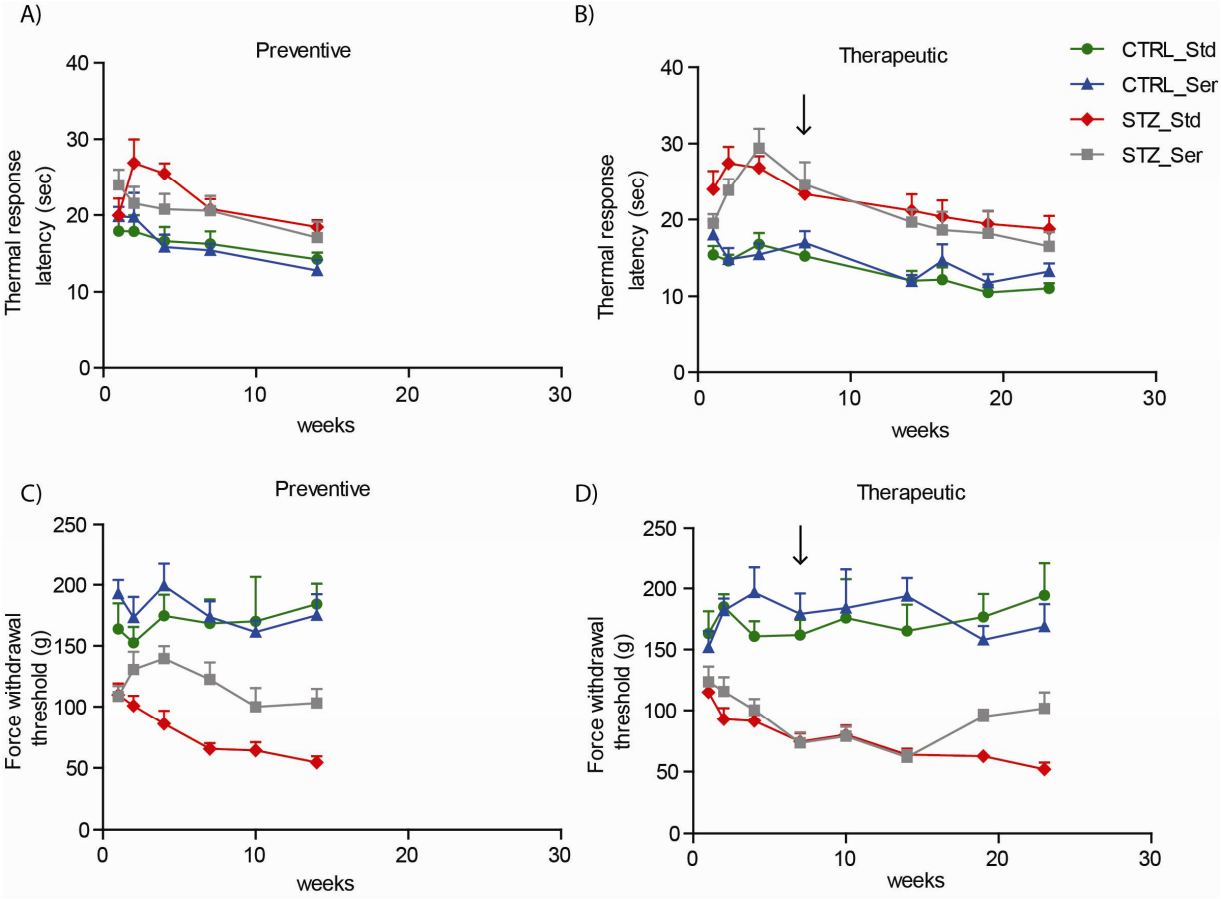

Figure 9. Time course of thermal and mechanical nociception. Thermal response latency (A-B) and force withdrawal threshold (C-D) were assessed for the preventive and the therapeutic groups at the beginning of the study and over time till the end the study. The values are expressed as mean ± SEM. Black arrows refer to the time of introduction of the serine-enriched diet to the respective groups in the therapeutic schedule. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet

In contrast, mechanical nociception was significantly impaired in the STZ groups (Fig. 9C-D, 10B). STZ rats on standard diet showed a significantly decreased withdrawal threshold (Fig 9 C-D) whereas serine-supplementation resulted in a significantly improved mechanical sensitivity in both, the preventive and the therapeutic schemes. In the preventive scheme, the force withdrawal threshold did not decrease in the serine treated STZ animals (103.2 g ±11.6 at week 14) while the STZ rats on standard diet showed a continuous decrease until the end of the study (54.6 g ± 5.0 at week 14). At the end of the preventive protocol, a significantly improved mechanical sensitivity was observed for the serine-treated STZ rats versus those on standard diet ($p < 0.01$). No difference was seen in the control animals. Similar results were obtained in the therapeutic scheme, mechanical sensitivity was not significantly different between the STZ groups before starting the serine supplementation in week 8 (74.5 g ± 6.6 in the STZ group on a standard diet and 73.4 g ± 8.4 in the STZ group on a serine-enriched diet).

Serine supplementation led to an improved mechanical sensitivity and the differences became significant at week 23 prior to sacrifice (52.0 g ± 5.4 on standard diet and 101.8g ± 13.0 on serine-enriched diet, $p < 0.001$) (Fig. 10B).

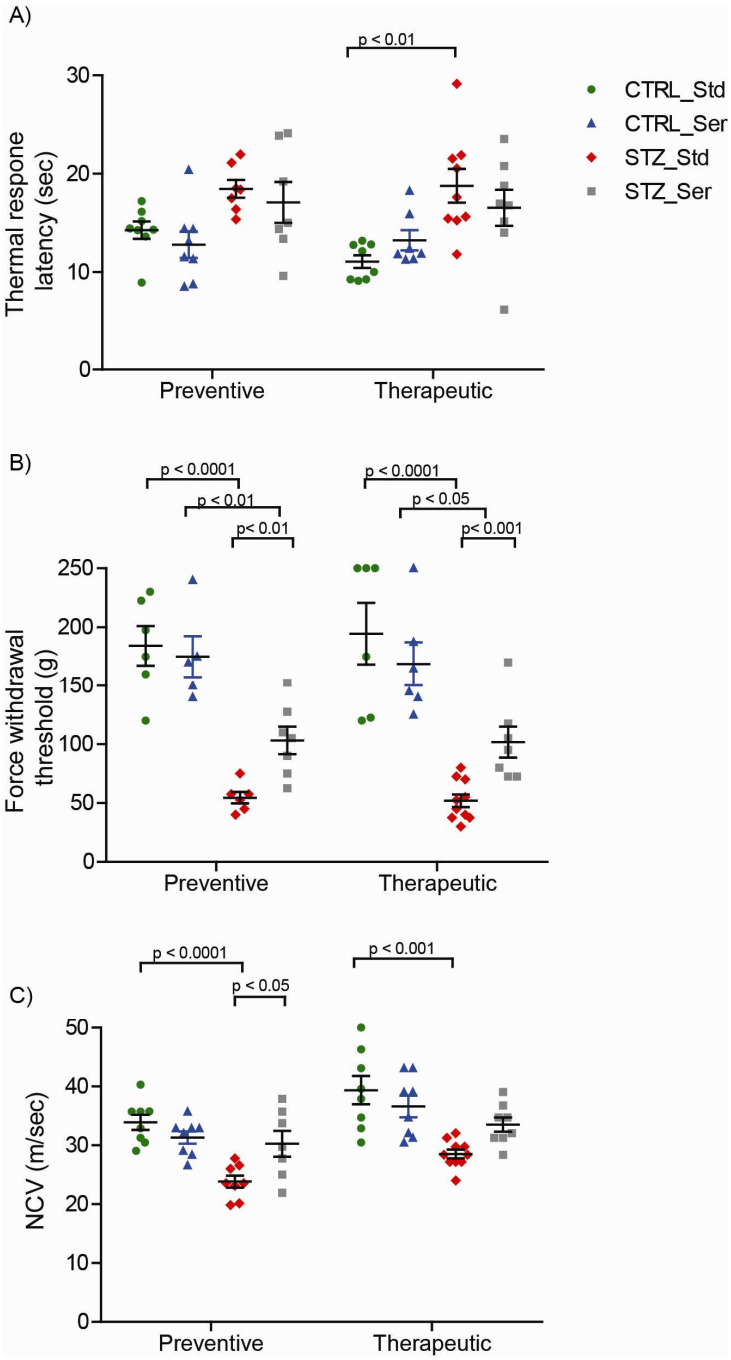

Figure 10. Neurobehavioral tests and nerve conduction velocity at the last time point before sacrifice. Thermal response latency (A) force withdrawal threshold (B) and sensory nerve conduction velocity (C) are shown for the preventive and the therapeutic groups at week 14 post-STZ injection for the preventive group and week 23 post-STZ injection for the therapeutic groups. The values are expressed as mean ± SEM. *p* values are calculated using ANOVA followed by the Bonferroni correction as mentioned earlier. For the force withdrawal threshold, the values were log-transformed before the calculation of the p values. CTRL_Std, control on standard diet, CTRL_Ser, control on serine diet, STZ _Std STZ on standard diet, STZ_Ser STZ on serine diet

Nerve conduction velocity (NCV) decreased significantly in the STZ groups on a standard diet compared to the controls (Fig. 10C) ($p < 0.0001$ comparing the STZ on standard diet to the control in the preventive schedule and $p < 0.001$ comparing the same groups in the therapeutic schedule).

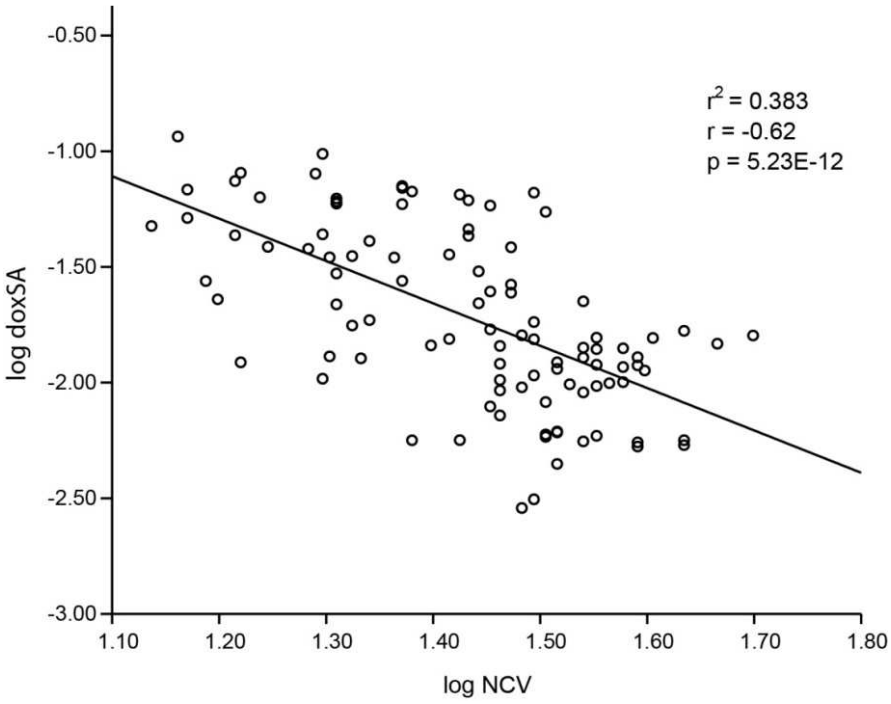

Figure 11. Scatter plot showing the correlation between the nerve conduction velocity (NCV) and plasma 1-deoxySA levels. The values from the different animal groups are used to investigate the correlation between plasma 1-deoxySA levels. The variables were log transformed since the control groups were skewing the normal distribution to the right. Pearson correlation coefficient and the asymptomatic *p* value are shown

STZ rats on the serine-enriched diet showed a significant improvement in the NCV for the preventive scheme (30.3 m/sec ± 2.2 in the STZ on a serine-enriched diet vs.23.8 m/sec ± 1.0 for the STZ on a standard diet, $p < 0.05$). No significant improvement in the NCV was observed in the serine-treated animals in the therapeutic schedule (Fig. 10C). Interestingly, plasma 1-deoxySA levels correlated inversely with the NCV values ($r = 0.62$, $p = 5.23 * 10^{-12}$) (Fig. 11).

Discussion:

The inherited neuropathy HSAN1 is associated with several mutations in SPT [23, 25] which lead to increased 1-deoxySL formation in the plasma and tissues of the affected carriers [27]. Elevated plasma 1-deoxySLs were also found in individuals with metabolic syndrome [31] and T2DM [28] despite the absence of mutations in the SPT. We demonstrated earlier that an oral L-serine supplementation effectively lowers 1-deoxySL levels in HSAN1 animal models and human patients. Serine-supplemented HSAN1 mice were protected and did not develop any neuropathic symptoms [27].

Here, we report that a serine supplementation is also effective in reducing the plasma 1-deoxySL levels and improves the neuropathy in diabetic STZ rats independent of changes in the plasma glucose levels. This was tested in preventive and therapeutic schemes. 1-deoxySLs were shown to be neurotoxic in vitro, impairing the length, number and branching of neurites in cultured dorsal root ganglia (DRGs) without directly affecting the viability of the neurons [25]. In addition, 1-deoxySLs inhibited neurite growth and induced cytotoxicity in primary dopaminergic neurons from rat ventral mesencephalon [32]. In both , the preventive and therapeutic schemes, we observed a significant reduction of the 1-deoxySL plasma levels upon serine supplementation which was associated with improved mechanical sensitivity, better NCV and increased neuronal Na/K activity (data no shown).. This suggests that the serine-mediated lowering of plasma deoxySLs might be connected to the improved nerve function in the serine-treated animals. Serine supplementation was reported earlier to improve neuronal differentiation and survival of embryonic chicken DRGs in-vitro [33] although the underlying mechanism was not clear. It was demonstrated that this effect was due to the serine itself and not mediated by a serine-to-glycine conversion and the activation of the glycine receptor [33] while other amino acids did not show this effect. It is noteworthy that neurons cannot synthesize L-serine due to the lack of 3-phosphgylcerate dehydrogenase (PHGDH), the rate limiting enzyme for serine biosynthesis. Neuronal cells depend, therefore, on the supply of serine from the supporting cells (e.g. glia and satellite cells). Astrocytes were shown to release L-serine which acts as a neurotrophic factor for fetal rat hippocampal neurons [34] and cerebellar Purkinje neurons [35], thereby promoting survival and phenotypic maturation. Interestingly, intra-peritoneal administration of L-serine was also shown to improve the paclitaxel induced peripheral neuropathy in a rat model [36].

Although 1-deoxSLs were elevated in the plasma of the diabetic rats, we did not detect 1-deoxySLs in the DRGs, sciatic nerve or spinal cord of the rats. This is in contrast to the results

from HSAN1 mice where 1-deoxySL showed a significant accumulation within the sciatic nerves [27, 37]. Although it is feasible that the total concentration of the 1-deoxySLs in the neuronal tissues of the STZ rats is below the detection limit of our method, it indicates that the neurotoxic effects are probably not caused by a toxic accumulation of these lipids within the nerve. A potential alternative explanation could be that 1-deoxySLs exert their neurotoxic effect by interfering with cellular signaling events. It has been reported that 1-deoxySA could bind and activate endothelial differentiation gene (EDG) receptors in cell culture [38, 39]. The EDG receptor family consists of several G protein coupled receptors that regulate various neuronal functions [40]. Another possible mechanism might be a 1-deoxySL- mediated modulation of protein kinase C (PKC) activity, since free sphingoid bases were shown to affect the PKC activity *in vitro* [41-43]. Interestingly, PKC is shown to be involved in the pathogenesis of diabetic microvascular complications including diabetic neuropathy [44] There is also increasing evidence from gene expression arrays and cell culture studies suggesting that 1-deoxySL interfere with the Rho-Rac signaling cascade which might impair neuronal cytoskeleton dynamics and growth cone formation [45] (data not shown).

For the moment, we cannot exclude that the observed beneficial effects of serine are mediated by other neurotropic mechanisms. Moreover, it is not clear yet whether the elevated plasma 1-deoxySLs in T2DM are causally involved in the pathogenesis of the diabetic neuropathy or simply indicators of the metabolic derailments in diabetes. Further mechanistic studies are, therefore, necessary to dissect the interplay between 1-deoxySLs formation and the protective effect of serine in the context of the diabetic neuropathy.

However, independent of the underlying mechanism we demonstrated that an oral serine supplementation could be an effective novel therapeutic approach in the treatment of diabetic neuropathy.

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Chapter 4

Fenofibrate but not Niacin Lowers Atypical Sphingolipids in Plasma of Dyslipidemic Patients *

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Contribution statement

AO did the lipid extraction, mass spectrometric analysis, the statistical analysis and wrote the manuscript. RB was involved in the study design, sample collection and patient characterization. IA assisted in the lipid extraction. AvE contributed to the data interpretation and critically revised the manuscript. TH was involved in data interpretation and supervision and critically revised the manuscript.

*** Manuscript in preparation**

Abstract:

Background:

Sphingolipid *de-novo* synthesis is typically initiated by the enzymatic condensation of palmitoyl-CoA and serine in a reaction catalyzed by the serine palmitoyltransferase (SPT). SPT can metabolize, in addition to these canonical substrates, other acyl-CoAs (C_{12}- C_{18}) and other amino acids such as alanine and glycine. This gives rise to a great variety of atypical sphingoid bases. In particular, the conjugation of alanine with palmitoyl CoA generates a novel class of 1-deoxyphingolipids (1-deoxySL) which lack the C_1 hydroxyl group. Pathologically elevated 1-dexySLs are associated with HSAN1 – a rare inherited neuropathy caused by several missense mutations in SPT. We demonstrated previously that the 1-deoxySLs are also significantly elevated in individuals with metabolic syndrome and type 2 diabetes mellitus As 1-deoxySLs show a strong correlation with plasma triglycerides (TG), we were interested to investigate the effect of the two triglyceride-lowering drugs, fenofibrate and niacin, on the sphingoid base profile in dyslipidemic patients.

Methods:

56 patients with primary hypercholesterolemia or mixed dyslipidemia were enrolled in a multi-center, open label, cross-over study to compare the effects of niacin and fenofibrate on the plasma sphingolipid profile. After an initial 2-to-6-week wash-out period, patients were randomized to either fenofibrate 160 mg/d for 6 weeks or niacin 0.5 g/d for 3 weeks followed by 1 g/d for 3 weeks. This treatment period was followed by a 4-week wash-out period and the same treatment scheme was repeated with crossed over groups. Plasma samples were taken before and at the end of each treatment/washout period and sphingoid base profile was quantified using LC/MS.

Results:

Fenofibrate and niacin had similar effects on lipoproteins and apolipoproteins apart from ApoAII. Both treatments increased HDL-C (+14.25 % and +10.92%, respectively) and apoAI (+7.7% and +5.82 %, respectively). The effect on increasing ApoAII was significantly higher for fenofibrate than for niacin (+ 27.95% for fenofibrate vs. 0.5% for niacin, $p < 0.001$). Fenofibrate lowered LDL-C (-15.99% vs. -6.9%), total cholesterol (-13.7% vs. -3.98%), ApoB (-18.4% vs, -7.11%) and triglycerides (-33.4% vs. -11.98%) compared to niacin. Besides these effects on the lipoprotein profile we observed a significant lowering of all

atypical sphingoid base species ($p < 0.001$). Fenofibrate treatment lowered 1-deoxySLs (-39.6 % for 1-deoxySO and -26.4% for 1-deoxySA), C_{20}-based sphingolipids (-28.0% for C_{20}SO and -24.6% for C_{20}SA), C_{19}SO (-24.4%), and the shorter chain backbone sphingolipids (-12.4 % for C_{17}SO, -11.6% for C_{16}SO). This effect was not observed with niacin. Interestingly, neither fenofibrate nor niacin showed any significant effect on the typical sphingolipids (C_{18}-based sphingolipids)

Conclusions:

Both, fenofibrate and niacin were effective in improving the lipoprotein profile in plasma of dyslipidemic patients. However, only fenofibrate but not niacin was also effective in lowering atypical plasma sphingolipids in dyslipidemic patients. None of the compounds did affect the typical C18-based sphingolipids.

Abbreviations:

1-deoxySA	1-deoxysphinganine
1-deoxySO	1-deoxysphingosine
1-deoxySLs	1-deoxysphingolipids
$C_{16\text{-}19}$	Carbon chain length (16 carbons -19 carbons)
HSAN1	Hereditary sensory and autonomic neuropathy type I
PPAR	peroxisome proliferator-activated receptors
SPT	Serine palmitoyltransferase
SA	Sphinganine
SO	Sphingosine

Introduction:

Pathologically altered plasma lipids are one of the major causes for cardiometabolic diseases and dyslipidemia remains one of the main therapeutic targets for the treatment of cardiovascular diseases and metabolic syndrome (MetS) [1, 2]. The best characterized lipid-modifying drugs include HMG-CoA reductase inhibitors, (statins), fibric acid derivatives (fibrates) and nicotinic acid derivatives (niacin) [3]. Fibrates have been identified as agonists for the peroxisome proliferator-activated receptors α (PPARα) [4], a member of the nuclear hormone receptor superfamily [5]. Three different subclasses of PPARs have been identified - PPARα, β / δ and γ [4, 6-8]. The activation of PPARα by fenofibrate has been shown to increase the expression of lipoprotein lipase which increases the release of fatty acids from the triglyceride-rich lipoproteins. Activation of PPARα stimulates also fatty acid uptake, activation and β-oxidation while it decreases fatty acid synthesis by down regulating acetyl-CoA carboxylase and fatty acid synthase. In addition, fenofibrate increases the expression of the apolipoproteins ApoAI and ApoAII, the two major lipoproteins of HDL, and down regulates ApoB and ApoCIII. Fibrates, therefore, improve the plasma lipid profile by decreasing plasma triglycerides and increasing HDL [9]. Niacin, on the other hand, exerts its functions through both receptor and non-receptor mediated routes. It binds to the membrane receptor GPR109A [10, 11] in adipocytes. This leads to the inhibition of lipolysis in adipose tissue by decreasing c-AMP concentration. Low c-AMP levels in adipocytes lead to decreased protein kinase A(PKA) activity which decreases the activity of the hormone sensitive lipase (HSL) [12]. This, in turn, decreases plasma free fatty acids and reduces triglyceride synthesis in the liver [12]. Niacin was also shown to lower the activity of cholesterol ester transfer protein (CETP) in ApoE*3 Leiden mice which were transgenic for the human CETP gene [13]. Moreover, non-receptor mediated effects of niacin have been reported [14] but the exact mechanisms leading to the improvements in the lipoprotein profile are not fully elucidated.

Plasma lipids are very heterogeneous in nature with > 600 distinct species. The most abundant classes of plasma lipids are sterols and glycerophospholipids reflecting approx. 50% and 30% of the total plasma lipids, respectively [15, 16]. Sphingolipids are present in plasma to a minor extent and represent about 4% of the total plasma lipids [15, 16]. However, more than 200 different sphingolipid species have been identified in plasma [15]. This diversity is due to the combination of different sphingoid base backbones with different *N*-linked fatty acid chains in combination with different headgroups [15, 17-19]. Sphingolipid biosynthesis is typically

initiated by the condensation of serine and palmitoy-CoA by the enzyme serine palmitoyltransferase (SPT). The product 3-ketosphinganine is rapidly reduced to sphinganine (C_{18}SA). C_{18}SA is subsequently *N*-acylated to dihydroceramides and finally converted to ceramides by the introduction of a C_4-C_5 double bond into the sphinganine backbone. Ceramides are building blocks for complex sphingolipids which are formed by attaching different headgroups to the C_1 hydroxyl group. In the degradation pathway, ceramides are hydrolyzed to sphingosine (C_{18}SO). C_{18}SA and C_{18}SO are usually referred to as sphingoid bases or sphingoid base backbones. Apart from the canonical substrates (serine and palmitoyl-CoA), SPT can also utilize other acyl-CoAs and other amino acids which results in the formation of a diverse class of atypical sphingoid bases. In particular, the use of alanine, instead of serine, forms the group of 1-deoxysphingolipds (1-deoxySL) which lack the C_1 hydroxyl group of the serine-based sphingolipids. Due to the missing C_1 -OH group, 1-deoxySLs can neither be converted to complex sphingolipids nor degraded by the canonical sphingolipid catabolism.

We showed previously that 1-deoxySLs are significantly elevated in patients with the metabolic syndrome [17] and T2DM [19]. A principal-component-analysis revealed that 1-deoxySLs are one of the most significant contributors to differentiate between MetS and healthy controls. Discriminant analysis revealed that 1-deoxySA and 1-deoxySO together with triglycerides and HDL-cholesterol are the best explanatory variables for non-diabetic metabolic syndrome [17]. In the previous studies, we also observed a strong positive correlation between 1-deoxySL plasma levels and triglycerides[17] which was not observed for the serine-based sphingolipids. This correlation is not obvious on a direct metabolic basis since 1-deoxySLs are not defined by their carbon chain but rather by their alanine moiety. This indicates a metabolic connection between TG, alanine and 1-deoxySL formation. In the current study, we investigated this relationship in more detail by testing whether the lowering of TGs by fenofibrate and niacin in dyslipidemic patients also affects the plasma sphingolipids and in particular the 1-deoxySL levels.

Methods:

Patients and study design:

56 patients with primary hypercholesterolemia or mixed dyslipidemia were enrolled into a multi-center, open label, cross-over study to compare the effects of niacin and fenofibrate on the plasma lipid profile (Fig. 1). Patients with a history of coronary heart disease were excluded. After an initial 2-to 6-week wash-out period, patients were randomized to either fenofibrate 160 mg/d for 6 weeks or niacin 0.5 g/d for 3 weeks followed by 1 g/d for 3 weeks. This treatment period was followed by a 4-week wash-out period and the same treatment scheme repeated with the crossed over groups.

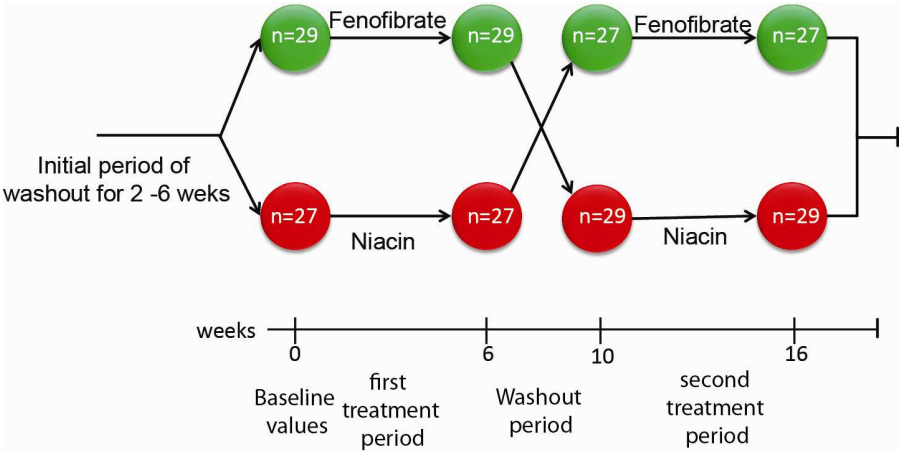

Figure 1. HiFUN study design. This was an open label randomized trial where patients with primary hypercholesterolemia and mixed dyslipidemia were enrolled. The initial wash-out period was applied to eliminate the effect of patients' past medication. Blood samples were collected at baseline, week 6, 10 and 16. The patients were treated for 6 weeks using either fenofibrate or niacin and then crossed over, with a wash-out period of 4 weeks in between. Green color represents the fenofibrate treatment while red represents the niacin treatment.

Fasting plasma samples were collected at baseline (week 0), after the end of the first treatment period (week 6), at the end of the wash-out period (week 10) and after the second treatment period following the cross over (week 16). The study was conducted by F. Hoffmann-La Roche Ltd. Basel, Switzerland. Plasma HDL-C, LDL-C, triglycerides, total cholesterol, ApoAI, ApoAII and ApoB were analyzed by Roche and collaborators. Clinical data were also provided by F. Hoffmann-La Roche Ltd. Basel

Sphingolipid analysis:

The sphingoid base profile was analysed as described before [17] with some modifications. Briefly, 0.5 ml methanol including 200 pmol of the internal standards d7-sphingosine and d7-sphinganine (d7SA, d7SO; Avanti Polar Lipids, Alabaster, Alabama, USA) was added

to100µl of plasma and extracted for 1h under agitation on a thermo-mixer at 37°C. Precipitated proteins were pelleted by centrifugation and the supernatant transferred to a new tube. For lipid hydrolysis, 75µl of methanolic HCl (1 N HCl and 10 M H_2O in methanol) was added to the supernatant and incubated for 16 hours at 65°C. This was followed by the addition of 100 µl of 10M KOH to neutralize the HCl and hydrolyze the phospholipids. To this mix, 625 µl chloroform was added. Then, 100 µl 2N ammonium hydroxide and 0.5 ml alkaline water were added to complete the phase separation. The mix was then vortexed and centrifuged at 16000 *g* for 5 minutes. After centrifugation, the upper phase was discarded and the lower organic phase was washed two to three times with alkaline water. Finally, the organic phase was dried under N_2 and kept at -20 °C freezer until analysis.

The sphingoid bases were separated on a C_{18} column (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, Montluçon, France) and analysed on a TSQ Quantum Ultra mass spec (Thermo, Reinach, BL, Switzerland). Each sample was measured as a singleton. Intra- and Inter-assay coefficient of variation (CV %) of the method was between 5% and 20%.

In this study 10 different sphingoid base backbones were analysed. The plasma levels of C_{16}SO, C_{17}SO, C_{18}SO, C_{18}SA, C_{18}SAdiene, C_{19}SO, C_{20}SO, C_{20}SA, 1-deoxySO and 1-deoxySA sphingoid bases were quantified.

Statistical analysis:

Data are expressed as mean ± SD or SEM as mentioned. Since the values for most of the measured variables did not follow a normal distribution even after log-transformation, non-parametric statistical methods were employed in the current study. Baseline and wash-out mean comparisons were performed and the *p* values were calculated using the Mann-Whitney U test followed by the Bonferroni correction (*p* value less than 0.001 was considered significant). To test the treatment effect, the mean comparison before and after each treatment was performed and Wilcoxon signed-rank test was used followed by the Bonferroni correction to calculate the *p* values. Statistical analysis was performed in SPSS 16.0 (IBM, Zurich, Switzerland)

Results:

Baseline characteristics of the study population are summarized in Table 1. The study subjects had normal levels for HDL-C (1.1 mol/l), and borderline-high LDL-C (4.03 mmol/l) levels according to the NCEP-ATPIII criteria [3] which resulted from the selection criteria that excluded patients with a history of coronary heart disease or its risk equivalents. In contrast, the average plasma triglycerides (2.49 mmol/l) and total cholesterol (6.26 mmol/l) were high according to the NCEP-ATPIII criteria [3]. No significant difference in the values for lipoproteins, apolipoproteins and sphingolipids was observed between baseline and after wash-out which excludes a carry-over effect of the two treatments.

Table 1. Baseline patient characteristics and clinical chemistry levels and after wash-out. Values are expressed as mean ± SD. *p* values are calculated using the Mann-Whitney U test to compare the baseline values to the values after the wash-out period. After the wash-out, none of the measured variables was significantly different from the baseline.

	Baseline (week 0)	After wash-out (week 10)	*p*
Age (years)	54.98 ± 9.75		
Weight (kg)	81.41 ± 11.65		
BMI	26.49 ± 2.24		
ApoAI (mg/dl)	136.43 ± 18.56	138.82 ± 16.54	0.57
ApoAII (mg/dl)	32.07 ± 4.26	33.57 ± 4.96	0.20
ApoB (mg/dl)	136.02 ± 18.18	139.55 ± 20.2	0.36
HDL-C (mmol/l)	1.1 ± 0.22	1.13 ± 0.22	0.50
LDL-C (mmol/l)	4.03 ± 0.57	4.22 ± 0.73	0.23
Total Cholesterol (mmol/l)	6.26 ± 0.65	6.45 ± 0.73	0.18
Triglycerides (mmol/l)	2.49 ± 0.99	2.67 ± 1.27	0.62
C_{16}SO (µmol/l)	24.61 ± 6.27	24.64 ± 6.83	0.76
C_{17}SO (µmol/l)	10.9 ± 2.51	10.94 ± 2.64	0.85
C_{18}SAdiene (µmol/l)	44.64 ± 8.37	45.86 ± 9.54	0.63
C_{18}SO (µmol/l)	136.94 ± 19.1	144.14 ± 29.75	0.33
C_{18}SA (µmol/l)	4.82 ± 1.3	5.55 ± 2.77	0.57
C_{19}SO (µmol/l)	3.65 ± 1.19	3.86 ± 1.57	0.87
C_{20}SO (µmol/l)	0.31 ± 0.09	0.35 ± 0.11	0.12
C_{20}SA (µmol/l)	0.04 ± 0.02	0.04 ± 0.02	0.88
1-deoxySO (µmol/l)	0.21 ± 0.1	0.23 ± 0.16	0.88
1-deoxySA (µmol/l)	0.12 ± 0.04	0.14 ± 0.07	0.56

Fenofibrate significantly increased ApoAI and ApoAII (Fig. 2A, B) and significantly lowered ApoB (Fig. 2C) whereas niacin had similar effects on ApoB and ApoAI but did not change ApoAII levels (Fig 2.A-C). In both groups plasma triglycerides, total cholesterol and LDL-C were significantly lowered whereas HDL-C was significantly increased (Fig. 2D-G).

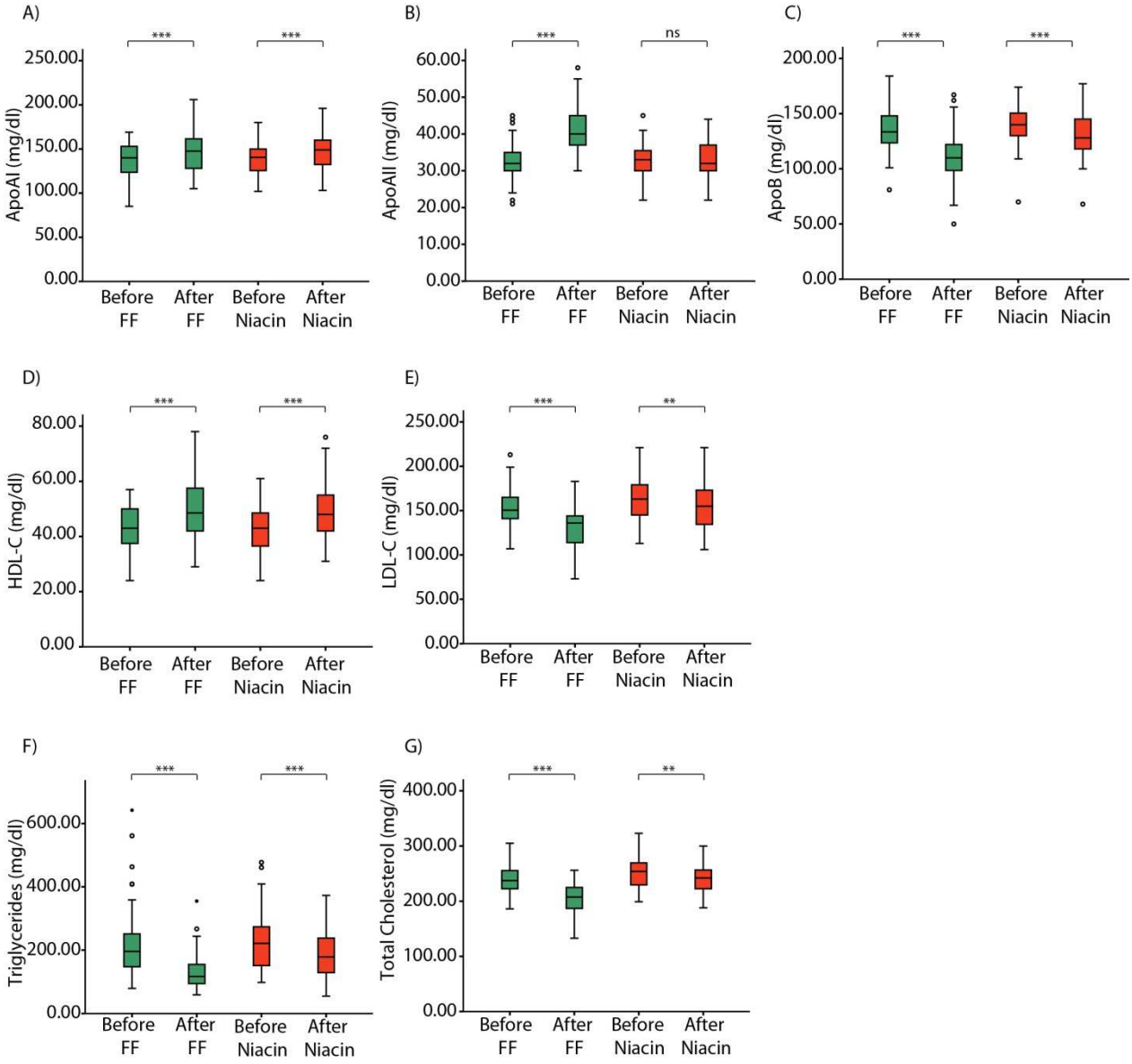

Figure 2. Fenofibrate and niacin treatment effect on the major apolipoproteins; ApoAI (A), ApoAII (B) and ApoB (C) and lipoproteins HDL-C (D), LDL-C (E), triglycerides (F) and total cholesterol (G). Box and whisker plots show the median and interquartile range of plasma lipoproteins and apolipoproteins in the patients before and after each treatment. *p* values were calculated using the Wilcoxon signed-rank test followed by the Bonferroni correction comparing the before and after treatment values. FF = fenofibrate, *** $p < 0.001$, ** $p < 0.01$, ns , non-significant,. Green color represents the fenofibrate treatment while red represents the niacin treatment.

Plasma sphingoid bases are usually *N*-acylated and conjugated to different headgroups, giving rise to the variety in sphingolipid species. In the current study, we focused our analysis on the sphingoid base backbone. To analyse the total sphingoid base profile of plasma sphingolipids, the lipids were extracted and hydrolysed to remove the *N*-acyl chain and head group. The here reported sphingoid bases concentrations therefore reflect the total concentration of sphingolipid species which are composed on this specific sphingoid base backbone.

In accordance with our earlier observations [17, 19] the correlation analysis (Table 2) showed a significant positive correlation of 1-deoxySL with triglycerides while the serine-based sphingoid bases correlated positively with LDL-C and total cholesterol (Table 2).

Table2 . Heat map of Spearman correlation coefficients for the analyzed plasma sphingolipids bases and the clinical chemistry and anthropometric variables in the current study. Bold font signifies a $p < 0.05$. The color code is showing the Spearman rho coefficient with the lowest value of -1 and the highest value of 1, Wcf, waist circumference, TC total cholesterol, TG triglycerides.

	C_{16}SO	C_{17}SO	C_{18}SAdiene	C_{18}SO	C_{18}SA	C_{19}SO	C_{20}SO	C_{20}SA	1-deoxySO	1-deoxySA
AGE	-0.05	-0.03	0.12	0.07	0.03	-0.07	0.12	0.07	0.07	0.05
Weight	**0.30**	**0.14**	-0.10	-0.05	**0.17**	**0.17**	0.06	**0.20**	**0.45**	**0.36**
Wcf	**0.18**	-0.01	-0.04	-0.09	**0.19**	0.06	0.05	**0.19**	**0.45**	**0.39**
BMI	**0.20**	0.12	0.03	-0.02	**0.14**	0.03	-0.02	**0.14**	**0.31**	**0.30**
ApoAI	**0.16**	0.02	0.08	0.00	0.01	-0.04	-0.05	0.01	**0.14**	0.13
ApoAII	-0.05	**-0.19**	-0.07	-0.06	-0.03	**-0.29**	**-0.33**	**-0.24**	-0.10	-0.03
ApoB	**0.37**	**0.41**	**0.44**	**0.41**	**0.18**	**0.37**	**0.46**	**0.43**	**0.34**	**0.29**
TC	**0.43**	**0.43**	**0.45**	**0.41**	**0.18**	**0.39**	**0.44**	**0.36**	**0.36**	**0.34**
LDL-C	**0.36**	**0.45**	**0.37**	**0.44**	**0.17**	**0.41**	**0.42**	**0.27**	0.13	**0.15**
HDL-C	0.05	0.00	0.10	0.05	-0.01	-0.05	-0.09	-0.11	-0.08	-0.05
TG	**0.19**	0.12	**0.17**	0.08	0.08	**0.18**	**0.27**	**0.34**	**0.53**	**0.44**

Spearman Rho						
-1	-0.5	-0.25	0	0.25	**0.5**	**1**

The plasma sphingoid base profile was analyzed before and after each treatment period (Fig. 3, 4). After fenofibrate treatment, we observed a lowering of all atypical sphingoid bases (C_{16}SO, C_{17}SO, C_{19}SO, C_{20}SO, C_{20}SA 1-deoxySO and 1-deoxySA) which was not seen after niacin treatment (Fig. 3).

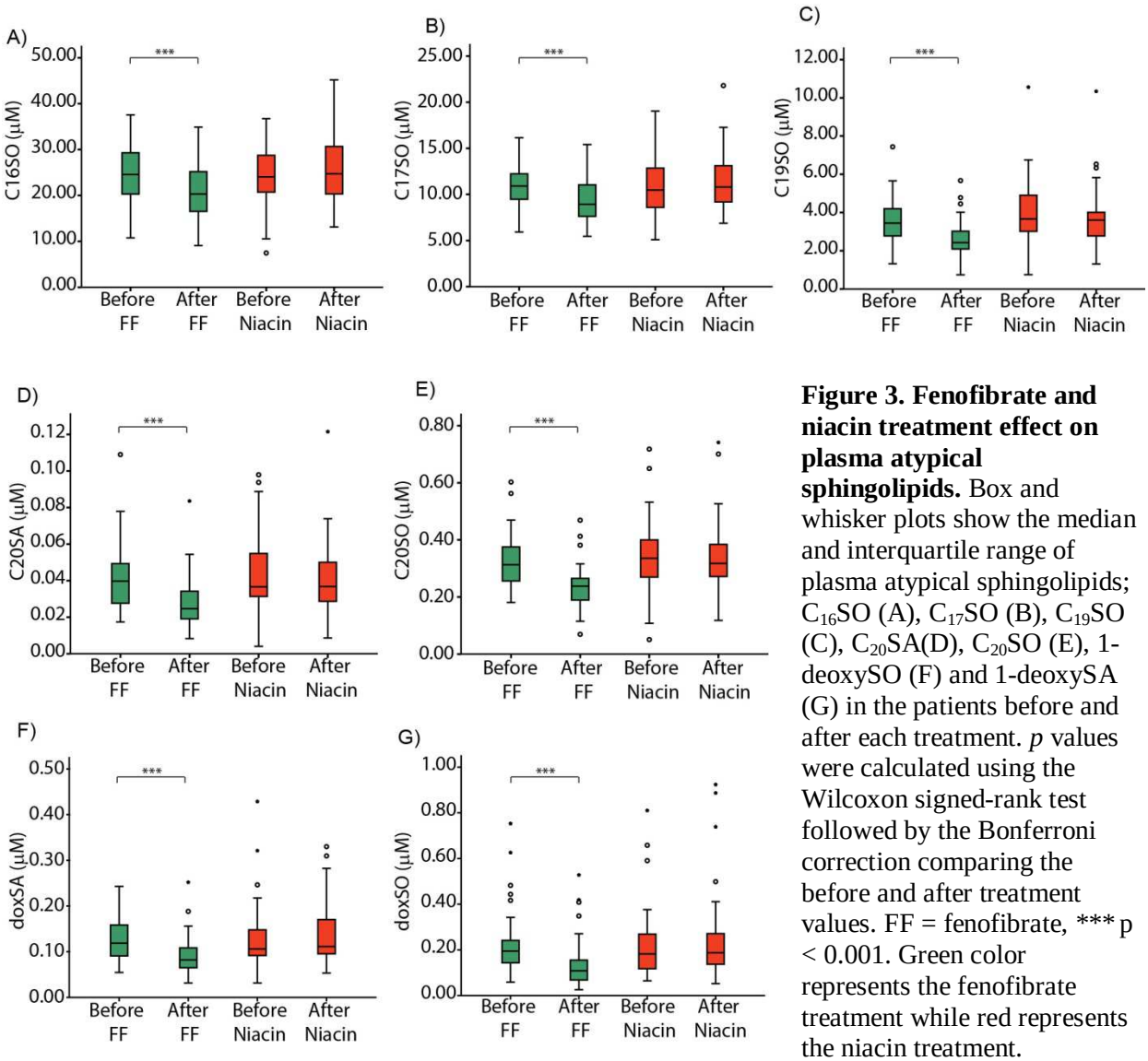

Figure 3. Fenofibrate and niacin treatment effect on plasma atypical sphingolipids. Box and whisker plots show the median and interquartile range of plasma atypical sphingolipids; C_{16}SO (A), C_{17}SO (B), C_{19}SO (C), C_{20}SA(D), C_{20}SO (E), 1-deoxySO (F) and 1-deoxySA (G) in the patients before and after each treatment. *p* values were calculated using the Wilcoxon signed-rank test followed by the Bonferroni correction comparing the before and after treatment values. FF = fenofibrate, *** $p < 0.001$. Green color represents the fenofibrate treatment while red represents the niacin treatment.

Neither fenofibrate nor niacin had any significant effect on the typical sphingoid bases (C18SO, C18SA and C18SAdiene) (Fig. 4), which represent together more than 84 % of the total sphingolipids in plasma. The effect of fenofibrate on the atypical sphingolipids was significantly different from that of niacin (Fig. 5A). Fenofibrate lowered 1-deoxySLs (-39.6 % for 1-deoxySO and -26.4% for 1-deoxySA), C_{20}-sphingoid bases (-28.0% for C_{20}SO and -24.6% for C_{20}SA), C_{19}SO (-24.4%) and also the shorter chain sphingoid bases (-12.4 % for C_{17}SO, -11.6% for C_{16}SO).

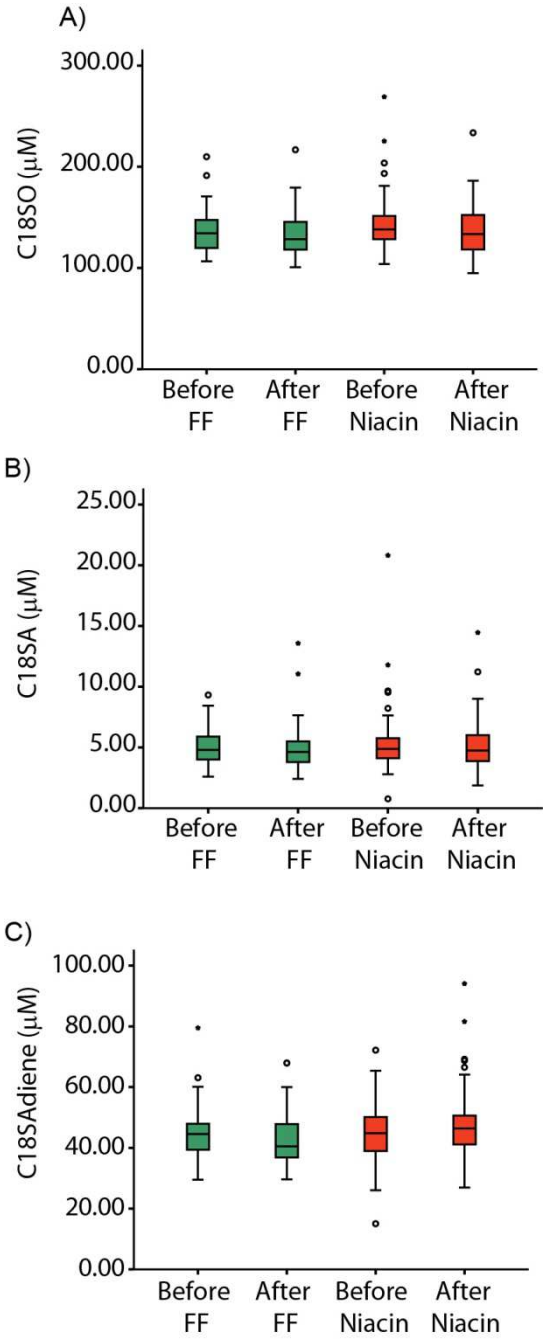

Figure 4. Fenofibrate and niacin treatment effect on plasma typical sphingolipids. Box and whisker plots show the median and interquartile range of plasma typical sphingolipids (C_{18}SO, C_{18}SA and C_{18}SAdiene) in the patients before and after each treatment. *p* values were calculated using the Wilcoxon signed-rank test followed by the Bonferroni correction comparing the before and after treatment values. FF = fenofibrate, *** $p < 0.001$. Green color represents the fenofibrate treatment while red represents the niacin treatment.

The effect of fenofibrate and niacin on the lipoprotein and apolipoprotein profile (Fig. 5 B) was comparable. Fenofibrate and niacin had similar effects on HDL-C (+14.25 % and +10.92%, respectively) and apoAI (+7.7% and +5.82 % for fenofibrate and niacin, respectively). The effect of fenofibrate on ApoAII was significantly different from that of niacin (+27.95 for fenofibrate vs. +0.5% for -niacin, $p < 0.001$). In general, fenofibrate was more effective in lowering LDL-C (-15.99% vs. -6.90%), total cholesterol (-13.7% vs. -3.98%), ApoB (-18.4% vs. -7.11%) and triglycerides (-33.4% vs. 11.42%) in comparison to niacin.

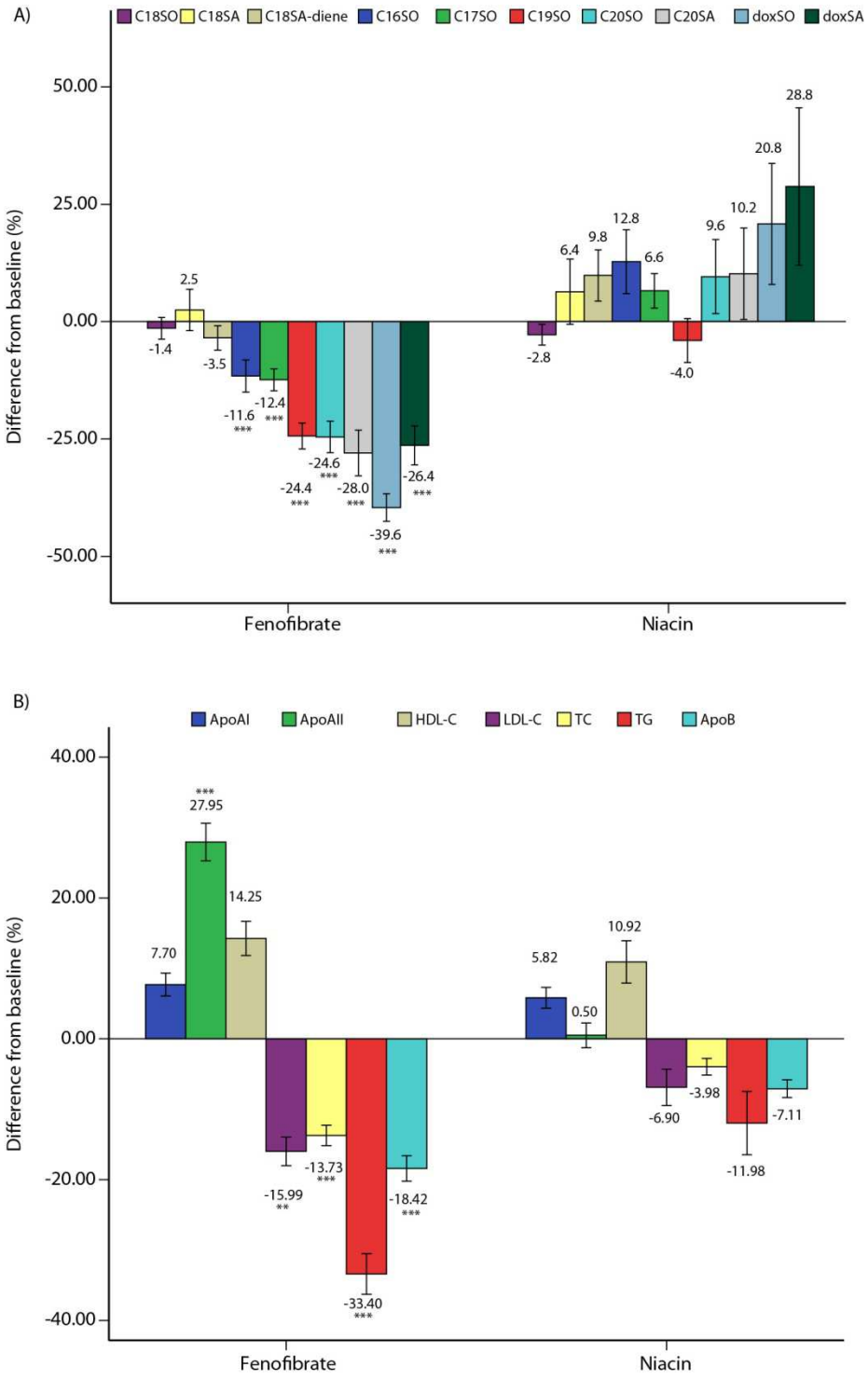

Figure 5. Difference from baseline due to fenofibrate or niacin treatment. Column plots show the mean difference from baseline in percent of the plasma sphingolipids (A) and lipoproteins and apolipoproteins (B) after each treatment**.** The data is shown as mean ± SEM. *p* values are calculated using Mann-Whitney U test followed by the Bonferroni correction comparing the fenofibrate to niacin effect. *** p <0.001, ** p <0.01

Discussion:

Atypical sphingolipids are formed by SPT through the incorporation of acyl-CoAs other than palmitoyl-CoA, or amino acids other than serine. In particular, the alternative use of alanine instead of serine results in the formation of a novel class of 1-deoxySLs. We have shown previously that pathologically elevated 1-deoxySLs are associated with the inherited neuropathy HSAN1 (hereditary sensory neuropathy type I) [20] which is caused by several missense mutations in SPT [21, 22]. Theses mutations lead to a shift in the substrate preference from serine to alanine and increase the 1-deoxySLs formation. 1-dexoxySLs are neurotoxic and were suggested to play a role in the pathogenesis of HSAN1 [20]. Interestingly, it was shown that an oral serine treatment suppresses the formation of 1-deoxySLs in HSAN1 animal models and humans [23]. Elevated plasma1-deoxySLs were also found in plasma of patients with metabolic diseases such as the metabolic syndrome and T2DM. This increased 1-deoxySL formation in metabolic diseases is not caused by mutations in SPT but is probably associated with a derailed carbohydrate and fatty acid metabolism. In two previous studies, we observed a strong correlation between plasma deoxySLs and triglyceride levels [17, 19]. In this study, we demonstrated that fenofibrate but not niacin is able to specifically lower atypical sphingoid bases in the plasma of patients with dyslipidemia. On the other hand, neither fenofibrate nor niacin showed any significant effect on the levels of typical sphingolipids. This effect on the atypical sphingoid bases is independent of the changes in the lipoprotein profile since both niacin and fenofibrate had comparable effects on triglycerides, LDL-C and total cholesterol (although with different efficacies). It is not clear at this point if this effect is due to PPARα activation or a non-PPARα mediated effect of fenofibrate although preliminary results from PPARα knock-out mice point to the involvement of PPARα (data not shown). In accordance with our earlier observations [17, 19], we showed in the current study a significant correlation between plasma triglycerides and 1-deoxySL levels However, this relationship is probably indirect since it is not the fatty acid but rather the conjugation of alanine, instead of serine, which defines the formation of 1-deoxySLs. This indicates that fenofibrate directly or indirectly might influence the use of the amino acid substrate by SPT. In contrast, neither fenofibrate nor niacin showed any significant effect on the plasma levels of the typical, serine based sphingoid bases (C_{18}SO, C18SA and C_{18}SAdiene).

The observation that 1-deoxySLs are neurotoxic and being elevated in HSAN1 [20] and T2DM, suggests a potential involvement of these lipids in the pathogenesis of the diabetic

sensory neuropathy. In post-hoc analyses from randomized clinical trials and longitudinal cohorts, fenofibrate has been associated with improvements in the diabetic neuropathy and other microvascular complications [24, 25]. Thus, it would be intriguing to investigate whether the lowering effect of fenofibrate on plasma 1-deoxySLs is associated with the observed improvement in neuropathy.

In this study, we have also shown that fenofibrate lowers other atypical sphingolipids (C_{16}, C_{17}, C_{19} and C_{20}-sphingoid bases). Primarily the SPTLC3 subunit [26, 27] , probably in conjunction with other subunits [28], might be responsible for the incorporation of different acyl CoAs, other than palmitoyl-CoA leading to the formation of these atypical sphingoid bases. It is not clear yet whether fenofibrate directly affects the expression or the function of these subunits or whether the observed effects are due to alterations in the substrate availability for SPT. Several lines of evidence suggest a connection between PPARs and sphingolipid metabolism. For example, in a rat partial hepatectomy model, activation of PPARα by bezafibrate decreased de novo sphingolipid synthesis [29]. In that model, it was suggested that the bezafibrate effect on SPT is mainly due to decreased substrate availability (palmitoyl-CoA) because of a decrease in plasma saturated fatty acids [29]. It was also shown that bezafibrate increases the activity of acid sphingomylinase, an enzyme hydrolyzing sphingomyelin into ceramide thereby increasing ceramide levels through the degradation pathway [30]. On the other hand, it was shown that pioglitazone, a PPARγ agonist, increased de novo sphingolipid activity in the rat hearts [31]. This effect was partly mediated through increased protein expression of SPTLC2 [31]. These observations point to a connection between PPARs and the expression of sphingolipid metabolizing enzymes. Interestingly, phytoceramide, phytosphingosine (PhytoSO) and C_{18}SA have been shown to be direct activators for PPARs, although to different extents. Several phytoSO analogues have been shown to activate PPARs without being selective to a specific subtype [33] and it was shown by affinity binding assay, that sphingosine and sphinganine were able to physically interact with PPARα which supports the notion that also free sphingoid bases can act as endogenous ligands for PPARs [34]. In contrast, ceramides and dihydroceramides failed to activate PPARs *in vitro* [32]. This indicates that the relationship between sphingolipids and PPAR activation could be bidirectional since some of the sphingolipid metabolites can bind and activate PPARs and PPAR activation might in return affect sphingolipid formation.

In summary, we have shown that fenofibrate but not niacin specifically lowers the atypical sphingolipids in the plasma of dyslipidemic patients without affecting the typical

sphingolipids. Further studies are needed to investigate this relationship in more detail and to explore the potential therapeutic use of PPARα activators for the treatment of the diabetic neuropathy.

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General conclusions and outlook

In this work, we evaluated the potential of plasma atypical sphingolipids to act as biomarkers in cardio-metabolic disease. We also evaluated some therapeutic strategies to modulate the levels of these atypical sphingolipids in plasma in the context of dyslipidemia and diabetic neuropathy.

We showed that 1-deoxysphingolipids (1-deoxySLs) were significantly elevated in the plasma of patients with metabolic syndrome and T2DM [1]. This was confirmed in three independent cohorts (total n = 524) [1, 2]. Multivariate analysis and receiver operating characteristic (ROC) curves revealed that 1-deoxySA and 1-deoxySO can discriminate between patients with metabolic syndrome and healthy controls better than other traditional biomarkers such as glucose, hypertension and HDL-C. Furthermore, we showed that 1-deoxySLs are independent predictors for the development of T2DM (Chapter 2). In particular, plasma 1-deoxySO had an adjusted odds ratio similar to HbA_{1c} and the presence of metabolic syndrome. The regression model containing 1-deoxySO, HbA_{1c} and the presence of metabolic syndrome showed a significantly higher ability (AUC of the ROC curve) to discriminate the incident T2DM group than each of the covariates alone. These findings establish the association between plasma 1-deoxySLs, T2DM and the metabolic syndrome. A limitation of the prospective T2DM study is the small number of events (incident T2DM =30). Thus, adjustment for all the potential confounders was not possible. Consequently, it would be important to reproduce the prospective potential of 1-deoxySLs in a larger prospective cohort. Eventually, an interventional randomized controlled trial has to be performed. In such a trial, patients with high and low plasma 1-deoxySLs should be compared in their response to different therapeutic options in T2DM [3, 4]. For example, in studies aiming at the prevention of T2DM (e.g. by life style intervention such as diet and exercise [5, 6] or by the use of therapeutic drugs like metformin[7]), the incidence of T2DM can be compared in patients with high and low plasma levels of 1-deoxySLs in different preventive schemes. This would show whether the clinical adoption of 1-deoxySA as a risk marker for T2DM development has a measurable beneficial effect on the patients' outcome.

Moreover, it would be interesting to explore the ability of plasma 1-deoxySLs as diagnostic or prognostic biomarkers for the microvascular and neurological complications of T2DM. This is of particular interest in the context of the diabetic neuropathy. This line of investigation is supported by our findings that plasma 1-deoxySLs are associated with both T2DM (Chapter1,

2) and the rare hereditary sensory neuropathy type I (HSAN1) [8]. Furthermore, *in-vitro* studies have shown that 1-deoxySLs are neurotoxic, affecting neurite length and the number of branching points [8] which suggests a potential role for 1-deoxySLs in the development of diabetic neuropathy.

Although we showed a robust association between 1-deoxySLs plasma levels, MetS and T2DM, a causal role in the pathogenesis of these diseases was not addressed. Further studies are necessary to investigate whether 1-deoxySLs are causally involved in the pathogenesis of T2DM or not. For example, it would be interesting to investigate whether 1-deoxySLs are involved in insulin resistance or in β-cell failure. Preliminary experiments showed that 1-deoxySA is toxic to pancreatic β cell lines and primary pancreatic islets (data not shown). The previous results should also be validated in T2DM animal models, e.g.in high-fat fed mice model. In these studies, the plasma 1-deoxySLs can be lowered by one of the strategies shown in the current work (fenofibrate or serine supplementation) and the development of pre-diabetes and impaired glucose tolerance can be monitored. This would show whether the lowering of 1-deoxySLs in plasma in these animal models would delay or prevent the progression to T2DM.

The mechanism through which plasma 1-deoxySLs are elevated in T2DM and MetS remains largely not understood. One possible mechanism could be an increase in intracellular alanine levels, which is the substrate for SPT to form 1-deoxySLs. Indeed, an increase in alanine flux into the liver in case of obesity and diabetes has been reported in early physiological human studies [9, 10]. Moreover, a high glycolytic flux in the liver of hyperglycemic patients is conceivable since the liver expresses mostly the insulin-independent glucose transporter 2 (GLUT2). This increased glycolytic flux might result in an increase in alanine as a side product of pyruvate accumulation when the TCA cycle capacity is exceeded. However the increased availability of alanine alone is not sufficient to increase 1-deoxySL formation in cultured cells (data not shown). This indicates that additional factors are required for the elevated 1-deoxySL formation in T2DM and MetS and warrants further investigation.

To our surprise, C_{20}SO, another atypical sphingoid base which is formed by the use of stearoly-CoA instead of palmitoyl-CoA, evolved as an independent risk predictor for cardiovascular events (Chapter 2). The predictive ability of this marker was significant even after the adjustment for other cardiovascular risk factors including the degree of coronary artery stenosis as graded by angiography. This implies that the C_{20}SO plasma levels add predictive power to angiography - the current gold standard for evaluating atherosclerosis.

These findings have also to be confirmed in another independent prospective cohort ideally with a larger number of follow-up events to validate the predictive ability of the marker.

We have previously shown that serine is effective in lowering plasma 1-deoxySLs in animal models and humans with hereditary sensory neuropathy type I (HSAN1) [14]. HSAN1 is associated with several missense mutations in SPT which were shown to increase the activity of SPT with alanine, thereby increasing 1-deoxySLs formation [8, 15, 16]. Given the similarity between diabetic neuropathy and HSAN1, and the finding that plasma 1-deoxySLs are elevated in both conditions, we investigated the effect of an oral L-serine supplementation on plasma 1-deoxSLs and the diabetic neuropathy in a streptozotocin-induced type 1 diabetes in rats. We showed that a serine-enriched diet significantly lowered the plasma 1-deoxySLs in STZ rats and improved the mechanical sensitivity and nerve conduction velocity. These findings showed that L-serine treatment might be a novel therapeutic option for the treatment of the diabetic neuropathy.

Interestingly, in contrast to the HSAN1 mice where 1-deoxySL accumulates in the sciatic nerves [8, 14], 1-deoxySLs were not detected in the sciatic nerves, DRGs, or spinal cords of the STZ rats (Chapter 3). This discrepancy argues against the concept that a toxic accumulation of 1-deoxySLs in the peripheral nerves contributes to the pathogenesis of diabetic neuropathy. Moreover, 1-deoxySLs could also not be detected in nerve biopsies from human patients with diabetic neuropathy (data not shown). On the other hand, there are several lines of evidence which support a potential role for 1-deoxySLs in the pathogenesis of neuropathy. In HSAN1, several single point mutations in the *SPT* lead to a neuropathy phenotype in humans and animal models that is mainly associated with increased 1-deoxySLs generation. Moreover, 1-deoxySA is shown to affect the neurite length and branching in chicken DRGs *in vitro,* which shows their neurotoxic properties. Furthermore, serine supplementation significantly lowered the plasma 1-deoxySLs in STZ and HSAN1 mice, improved the neuropathy in the STZ rats and precluded the development of the neuropathy in the HSAN1 mice whereas alanine supplementation increased the plasma 1-deoxySL in HSAN1 mice and exacerbated the neuropathy [14].

To resolve this discrepancy, it is important to address two major questions: First, whether elevated plasma 1-deoxySLs are sufficient to induce a neuropathy *in vivo,* and second, whether the above-mentioned beneficial effects of serine in neuropathy are mediated solely through the lowering of plasma 1-deoxySL. To address these points, several strategies could be employed. For example, a specific transient elevation of plasma 1-deoxySLs by the

injection of a specific 1-deoxySL formulation (e.g. single or multiple injections by intravenous, intra-peritoneal or intra-thecal route) in animals would allow the evaluation of the short-term effects of 1-deoxySL elevation in plasma or cerebrospinal fluid (CSF). Moreover, a viral-mediated delivery of a 1-deoxySL-producing mutant of SPT (e.g. an HSAN1 associated mutation) to the liver of WT animals could help to address the systemic effects of elevated plasma1-deoxySLs. Another approach would be to inject the serine-supplemented STZ rats with a specific 1-deoxySL formulation to re-induce the neuropathy, to exclude that the protective effect of serine is mediated by a general neurotrophic action independent if its ability to lower the plasma1-deoxySLs.

In the clinical studies, we observed a consistent positive correlation between plasma 1-deoxySLs and triglycerides (TG). 1-deoxySLs are transported mainly in VLDL and LDL[2], and correlate positively and strongly with TGs which are also enriched in VLDL and LDL. Therefore, we hypothesized that the increase of plasma 1-deoxySL in MetS and T2DM might be due to the increase in its lipoprotein containing particles. This hypothesis was evaluated in an interventional clinical trial in which the effects of fenofibrate and niacin, two triglyceride-lowering drugs, on plasma sphingolipids were tested in dyslipidemic patients. We showed that fenofibrate but not niacin significantly lowered the atypical sphingolipids (C_{16}SO, C_{17}SO, C_{19}SO, C_{20}SO, C_{20}SA 1-deoxySO and 1-deoxySA) in the plasma without any significant effect on the typical sphingolipids (C18SO, C18SA and C18SAdiene). Interestingly, both fenofibrate and niacin lowered plasma triglycerides as expected. This demonstrates that the atypical sphingolipids (including 1-deoxySLs) in the plasma can be lowered by fenofibrate but not by niacin, although both drugs showed comparable effects on TG levels and the lipoprotein profile. This refutes our initial hypothesis and instead indicates that the increase in plasma 1-deoxySL in T2DM and MetS is probably due to other reasons and not merely a bystander of the increase in the production or excretion of the 1-deoxySL-containing lipoproteins. This also shows that the 1-deoxySL-lowering-effect of fenofibrate might be related to its major mode of action, i.e. PPARα activation. This aspect could be addressed in more detail using PPARα knock-out (KO) animal model. In these experiments, the effect of fenofibrate on plasma 1-deoxySLs could be tested by feeding KO and WT animals a high-fat diet, which increases the plasma 1-deoxySLs, together with fenofibrate. If fenofibrate lowers plasma 1-deoxySLs only by a PPARα-dependent mechanism, then this effect should not be observed in the PPARα KO model. This would elucidate whether the effect of fenofibrate on plasma 1-deoxySLs is mediated by PPARα or by other off-target effects. In a similar approach, potent specific agonists and antagonists of PPARα can be added to cell culture

models of increased 1-deoxySA generation (e.g. cell lines incubated with high palmitate or high glucose or both). This would also help dissect out the role of PPARα, if any, in regulation of 1-deoxySL formation.

Interestingly, fenofibrate lowered also the other atypical sphingolipids which are usually generated by the use of different acyl-CoAs. Little is known about the regulation of SPT acyl-Co substrate specificity. Our lab has recently identified a new subunit of SPT, SPTLC3, which shifts the substrate specificity to different acyl-CoAs other than palmitoyl-CoA [11, 12]. Other subunits associated with the SPT enzyme have also been suggested to regulate the acyl-CoA substrate specificity of SPT [13]. In this context, it will be interesting to investigate whether fenofibrate affects the expression or the activity of these subunits e.g. by adding fenofibrate or other specific PPARα agonist to cell lines that naturally express high levels of these subunits or by similar strategies.

In summary, we have identified some of the promising potentials of atypical sphingolipids as novel biomarkers and therapeutic targets in T2DM, MetS and cardiovascular disease. Although most of these atypical sphingolipids are usually present in minor concentrations in plasma, they might not be minor regarding their clinical significance. Thus, this work, hopefully, paves the way for further studies, both clinical and mechanistic, to further explore their full potential.

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Publications:

***Published**:*

1. Bertea M, Rutti MF, **Othman A**, Marti-Jaun J, Hersberger M, von Eckardstein A, Hornemann T, **Deoxysphingoid bases as plasma markers in diabetes mellitus**. *Lipids Health Dis* 9:84, 2010

2. Karuna R, Park R, **Othman A**, Holleboom AG, Motazacker MM, Sutter I, Kuivenhoven JA, Rohrer L, Matile H, Hornemann T, Stoffel M, Rentsch KM, von Eckardstein A: **Plasma levels of sphingosine-1-phosphate and apolipoprotein M in patients with monogenic disorders of HDL metabolism**. *Atherosclerosis* 219:855-863, 2011

3. Steiner C, **Othman A**, Saely CH, Rein P, Drexel H, von Eckardstein A, Rentsch KM: **Bile acid metabolites in serum: intraindividual variation and associations with coronary heart disease, metabolic syndrome and diabetes mellitus**. *PLoS One* 6:e25006, 2011

4. Othman A, Rutti MF, Ernst D, Saely CH, Rein P, Drexel H, Porretta-Serapiglia C, Lauria G, Bianchi R, von Eckardstein A, Hornemann T: **Plasma deoxysphingolipids: a novel class of biomarkers for the metabolic syndrome?** *Diabetologia* 55:421-431, 2012

Submitted:

5. Ernst D, Murphy S.M., Sathiyanadan K., Wei Y., **Othman A.**, Papa R., Laura M., Liu Y., Blake J., Donaghy M., Winer J., Houlden H., von Eckardstein A., Reilly M.M. and T. Hornemann T. **Novel HSAN1 mutation in Serine-Palmitoyltransferase resides at a putative SPTLC2 phosphorylation site that is involved in regulating substrate specificity**, *submitted*

In preparation:

6. Othman A., Saely C.H., Drexel H., A. von Eckardstein A., and Thorsten Hornemann T., **Plasma Sphingolipid Profiling Reveals Novel Distinct Biomarkers for Predicting Cardiovascular Disease and Type 2 Diabetes Mellitus**, *in preparation*

7. Othman A., Benghozi R., Alecu I., von Eckardstein A., and Hornemann T., **Fenofibrate but not Niacin Lowers Atypical Sphingolipids in Plasma of Dyslipidemic Patients**, *in preparation*

8. Othman A., Bianchi R., Alecu I., Wei Y., Porretta-Serapiglia C., Lauria-Pinter G., von Eckardstein A., and Hornemann T., **Oral L-Serine Supplementation Lowers Plasma 1-Deoxysphingolipids in Experimental Diabetic Rats and Improves Diabetic Neuropathy,** *in preparation*

Oral Presentations:

1- The 6th international Charleston Ceramide Conference (CCC), March 16- 20, 2011 in Villars, Switzerland

2- The 10th Day of Clinical Research, June 9, 2011 in Zurich, Switzerland

3- The 5th joint meeting of the French, German and Swiss atherosclerosis Societies, June 16-18, 2011 in Avignon, France

4- The 6th annual retreat of the competence center for systems physiology of complex diseases, May 30 – 31, 2011 in Kartause Ittingen, Switzerland

5- AGLA & Cardiovascular Biology Meeting Jan 10-11, 2013 in Bern, Switzerland

Poster Presentations:

1- The 6th Congress of Central European Diabetes Association, June 30 -July 2, 2011 in Zurich, Switzerland

2- The 7th Symposium of the ZIHP, August 26, 2011 in Zurich, Switzerland

3- The 34th European Lipoprotein Club annual conference, September 5-8, 2011 in Tutzing, Germany

4- The IX Sphingolipid Club Meeting, Sept 28- Oct 1, 2011 in Favignana, Italy

5- AGLA & Cardiovascular Biology Meeting, March 8, 2012 in Zurich, Switzerland

6- Gordon research conference on Glycolipid & Sphingolipid Biology, April 22-27, 2012 in Lucca (Barga), Italy

7- The 8th Symposium of the ZIHP, August 24, 2012in Zurich, Switzerland

8- EMBO|EMBL symposium, Diabetes and Obesity, September 13-16 2012, EMBL Heidelberg, Germany

Curriculum vitae

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Name:	Alaa Mohamed Ahmed Othman
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Education:

2010 -2013:	PhD studies, University Hospital Zurich, University of Zurich, Zurich, Switzerland)
2006 -2009:	MSc. Master of Biochemistry and Molecular Biology, Bremen University, Bremen, Germany
1997-2004:	M.B.B.Ch, Bachelor of Medicine and Surgery, Cairo University, Faculty of medicine, Cairo, Egypt (equivalent to *MD* in German speaking countries)
1994 -1997:	High school, Toukh, Kalubyyah, Egypt.

Research experience:

2010 – 2013	PhD student on "role of atypical sphingolipids in diabetes mellitus and its complications" (P.D. Dr. Thorsten Hornemann, University of Zurich, Zurich, Switzerland)
2009-2010:	Research assistant in biochemical engineering "directed evolution of denaturating-agent-resistant DNase for RNA purification. (Prof. U. Schwaneberg, Jacobs University Bremen, Germany)
2008- 2009:	Master thesis "a quantification system for cellular uptake of boronated liposomes and the kinetics and possible mechanisms of uptake". (Prof. D. Gabel, Bremen University, Bremen, Germany)

2007-2008: Lab rotation “developing a model to predict water solubility of ionic liquids” (Dr. J. Ranke, Bremen University, Bremen, Germany)

2007: Lab rotation “developing a model for prediction of DNA fragmentation after alkaline lysis of a DNA template including AlphaS nucleotides”. (Prof. U. Schwaneberg, Jacobs University, Bremen, Germany)

Work experience:

2006 - 2013: Lecturer Assistant, medical biochemistry department, faculty of medicine, Cairo University, Cairo, Egypt “*on sabbatical leave till March 2013*”

2005- 2006: Pediatric Resident, Cairo University Hospitals Abu-Elrish Pediatric Hospital, Cairo University Hospitals, Cairo, Egypt

2004-2005: House Officer “Arzt im Praktikum AIP”, Departments of Surgery, Internal Medicine, Gynecology and Obstetrics, Pediatrics, Critical Care, Psychiatry, Emergency medicine and Anesthesia, Cairo University Hospitals, Cairo, Egypt